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(54) Title: ERYTHROPOIETIN WITH ALTERED BIOLOGICAL ACTIVITY

ARGININE 103 IS ESSENTIAL FOR EPO'S ACTIVITY
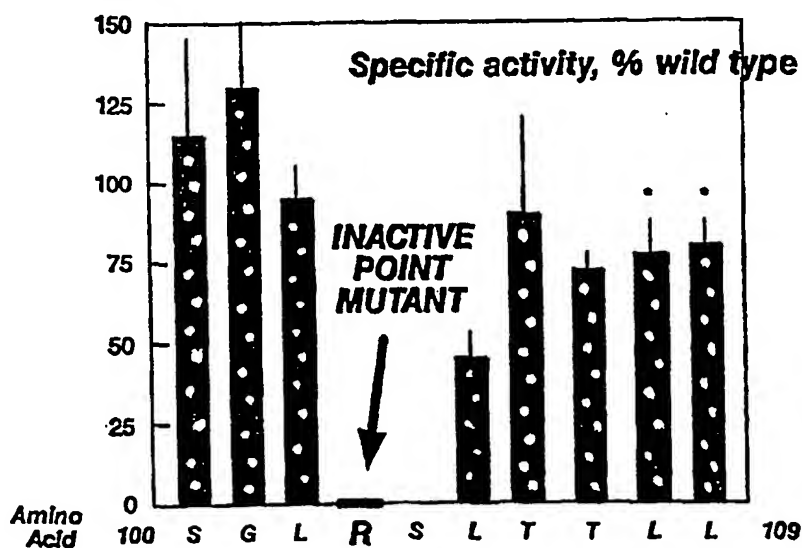
(57) Abstract

The invention relates to DNA encoding modified, secretable erythropoietin proteins whose ability to regulate the growth and differentiation of red blood cell progenitors are different from the wildtype recombinant erythropoietin. The invention also relates to methods of modifying or altering the regulating activity of the secretable erythropoietin proteins and the use of the modified secretable erythropoietin proteins, for example, in *in vivo* therapeutics.

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ERYTHROPOIETIN WITH ALTERED BIOLOGICAL ACTIVITY

RELATED APPLICATIONS

This application claims priority to U.S. Serial No.
09/017,631, filed on February 3, 1998, which is a
5   continuation-in-part application of U.S. Serial No.
08/808,881 which was filed on February 28, 1997 which is
a divisional of U.S. Serial No. 08/383,743 filed
February 2, 1995 issued as U.S. Patent No. 5,614,184 on
March 25, 1997, which is a continuation-in-part
10   application of U.S. Serial No. 08/113,080, filed August
26, 1993, now abandoned, which is a continuation-in-part
application of U.S. Serial No. 07/920,810, filed July
28, 1992, now abandoned. The teachings of these related
applications are incorporated herein by reference.

15   BACKGROUND OF THE INVENTION

The glycoprotein hormone erythropoietin regulates
the growth and differentiation of red blood cell
(erythrocyte) progenitors. The hormone is produced in
the fetal liver and adult kidney. Erythropoietin
20   induces proliferation and differentiation of red blood
cell progenitors through interaction with receptors on
the surface of erythroid precursor cells.

Several approaches have been employed to identify
those features of the protein that are relevant to its
25   structure and function. Examination of the homologies
among the amino acid sequences of erythropoietin

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proteins of various species has demonstrated several highly conserved regions (McDonald, J.D., *et al.*, *Mol. Cell. Biol.* 6: 842-848 (1986)).

5   Oligonucleotide-directed mutagenesis has been used to prepare structural mutants of erythropoietin, lacking specific sites for glycosylation. Studies indicate that N-linked carbohydrates are important for proper biosynthesis and/or secretion of erythropoietin. These studies also show that glycosylation is important for *in*

10 *vivo*, but not *in vitro*, biological activity. (Dube, S., *et al.*, *J. Biol. Chem.* 263:17516-17521 (1988); Yamaguchi, K., *et al.*, *J. Biol. Chem.* 266:20434-20439 (1991); Higuchi, M., *et al.*, *J. Biol. Chem.* 267:7703-7709 (1992)).

15   Studies with monoclonal anti-peptide antibodies have shown that the amino terminus and the carboxy-terminal region (amino acids 152-166) of erythropoietin may be involved with biological activity. It has also been demonstrated that antibodies to amino acids 99-119

20 and 111-129 block the hormone's biological activity, apparently by binding to two distinct non-overlapping domains (99-110 and 120-129). (Sytkowski, A.J. and Donahue, K. A., *J. Biol. Chem.* 262:1161-1165 (1987)). Thus, it was hypothesized that amino acids 99-129 were

25 important in the formation of a functional region involved in receptor recognition, either through forming a necessary component of the protein's tertiary structure or through direct participation in receptor binding, or both.

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Preliminary experiments suggested that alterations in localized secondary structure within the 99-129 region resulted in inactivation of erythropoietin. Therefore, a possible structural role for amino acids
5   99-129 has been postulated. Recently, a series of experiments indicated that amino acids 99-110 (Domain 1) play a critical role in establishing the biologically active conformation of human erythropoietin. (Chern, Y., et al., Eur. J. Biochem. 202:225-229 (1991)).

10 These Domain 1 mutants, in which a group of three amino acids was deleted and replaced by two different amino acids, were found to be biologically inactive. Furthermore, these mutations in Domain 1 inhibited the secretion of the mutant erythropoietin into cell culture
15 medium. (Chern, Y., et al., Eur. J. Biochem. 202:225- 229 (1991)). Inhibition of secretion in mammalian cells is consistent with a profound structural change of the polypeptide hormone. Profound structural changes could significantly affect the ability of the hormone to
20 interact with its cognate receptor. Thus, these mutant erythropoietin polypeptides are not suitable for elucidating the structure/function relationship that exists between erythropoietin and its cellular receptor. Nor are these mutants suitable erythropoietin
25 antagonists for use, for example, in therapeutic treatment of polycythemias, or over production of erythropoietin. Thus, it would be beneficial to precisely determine which amino acids are critical to the erythropoietin polypeptide to maintain a stable,
30 biologically active conformation which retains its

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secretable properties and its ability to bind to the
erythropoietin receptor.

Moreover, the precise determination of critical
amino acid residues would be useful to alter the
5 biological activity of erythropoietin, either decreasing
or increasing one or more biological properties of the
protein.

SUMMARY OF THE INVENTION

The present invention relates to isolated DNA
10 encoding mutated erythropoietin proteins which have
altered biological activity, yet retain their secretable
properties (i.e., secretable erythropoietin proteins).

In one embodiment, the present invention relates to
isolated DNA encoding secretable erythropoietin proteins
15 which have at least one amino acid residue in Domain 1
which differs from the amino acid residue present in the
corresponding position of wildtype erythropoietin and
which have altered ability to regulate the growth and
differentiation of red blood cell progenitors. Domain 1
20 of the mutants described herein refers to the amino
acids which correspond to amino acids 99-110 (SEQ ID NO:
1) of the wildtype recombinant erythropoietin. Altered
ability is defined as ability different from that of the
wildtype recombinant erythropoietin ability to regulate
25 the growth and differentiation of red blood cell
progenitors. As used herein, altered ability to
regulate the growth and differentiation of red blood
cell progenitor cells refers to biological activity
different from wildtype recombinant erythropoietin

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activity (i.e., altered biological activity relative to
wildtype recombinant erythropoietin activity). The
mutated erythropoietin proteins of the present invention
can be secreted in homologous and heterologous
5   expression systems. For example, the mutated
erythropoietin proteins of the present invention can be
secreted in mammalian, bacterial or yeast expression
systems.

       The present invention also relates to the modified
10 secretable mutant erythropoietin proteins encoded by the
isolated DNA described above. These modified secretable
erythropoietin proteins have altered biological
activities. For example, the modified secretable mutant
erythropoietin may have decreased ability relative to
15 wildtype erythropoietin protein to regulate growth and
differentiation of red blood cell progenitor cells. As
used herein, decreased ability to regulate growth and
differentiation of red blood cell progenitor cells is
also referred to as decreased biological activity
20 relative to wildtype erythropoietin activity. Wildtype
erythropoietin activity is also referred to herein as
biological activity of wildtype erythropoietin.
Alternately, a modified secretable mutant erythropoietin
protein described herein may exhibit increased heat
25 stability relative to wildtype erythropoietin protein.

       The modified erythropoietin proteins described
herein comprise an amino acid sequence with at least one
amino acid residue different from the amino acid residue
present at the corresponding position in Domain 1 in the
30 wildtype erythropoietin. These erythropoietin proteins

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are referred to as modified secretable human recombinant erythropoietin proteins having altered ability (i.e., decreasing or enhancing ability) relative to wildtype erythropoietin protein to regulate the growth and

5   differentiation of red blood cell progenitors.

The term modified, as used herein, includes substitution of a different amino acid residue, or residues, as well as deletion or addition of an amino acid residue, or residues.

10   Until the present invention, mutations within the erythropoietin sequence which result in the alteration of biological activity have also frequently resulted in a concurrent loss of secretability of the protein from transfected cells. This loss of secretability is

15   consistent with a loss of structural integrity. (Boissel, J-P. and Bunn, H. F., "*The Biology of Hematopoiesis*", pp. 227-232, John Wiley and Sons, New York (1989)). Now, the sites critical to the maintenance of a stable, biologically active

20   conformation have been identified by means of oligonucleotide-directed mutagenesis and have been found to occur in Domain 1 (amino acids 99-110) (SEQ ID NO: 1) of human recombinant erythropoietin. Modifications of the wildtype erythropoietin have been made and the

25   encoded erythropoietin proteins have been expressed. The resulting mutant erythropoietin proteins described herein have altered erythropoietin regulating activity, as demonstrated in the art-recognized bioassay of Krystal, G., *Exp. Hematol.* 11:649-660 (1983). Activity

30   of the resulting erythropoietin proteins has also been

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evaluated by commercially available radioimmunoassay
protocols.

In particular, the arginine 103 site is essential
for erythropoietin activity. As shown herein,
5   replacement of the arginine 103 by another amino acid
results in a modified erythropoietin with significantly
decreased biological activity relative to wildtype
erythropoietin activity. Modifications at this site, as
well as other sites within Domain 1, can similarly be
10 made to enhance regulating activity, as well as to
decrease, or reduce regulating ability.

In another embodiment, the present invention
relates to mutant proteins described herein that
comprise modified erythropoietin proteins produced by
15 alterations in the 5' and/or 3' noncoding regions of the
wildtype gene in addition to mutations in coding
regions. Hereinafter, the term modified erythropoietin
variant protein will be used to describe these
molecules.

20   These recombinant variant proteins can have altered
biological activity. Altered biological activity is
defined herein as activity different from that of the
wildtype or recombinant protein (e.g., the activity of
modified erythropoietin variant proteins to regulate the
25 growth and differentiation of red blood cell
progenitors). Modified erythropoietin variant proteins
can have increased activity relative to wildtype
erythropoietin to regulate growth and differentiation of
red blood cell progenitor cells. Alternatively, the
30 erythropoietin variant proteins can have decreased

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biological activity relative to the wildtype
erythropoietin.

Mutations in noncoding regions of the gene (e.g.,
5' untranslated regions or UTR) can lead to differences
5   in RNA translation as described, e.g., in Schultz, D.E.,
et al., J. Virol. 70:1041-1049, 1996; Kozak, M., J. Mol.
Biol. 235:95-110, 1994; and Kozak, M., J. Biol. Chem.
266:19867-19870, 1991. For example, as described in
detail in Example 4, computer modeling can be used to
10   predict differences in RNA secondary structure (e.g.,
free energy of loops and base pairs) following
nucleotide alterations in 3' and 5' UTR of the
erythropoietin gene. Although secondary structure
changes in EPO RNA, following mutations in the 5' or 3'
15   UTR, are used as the specific example, it is understood
that the instant invention described herein can be used
to produce any suitable polypeptide variant protein. As
used herein, the term mutation refers to any alteration
in the nucleic acid sequence encoding a polypeptide
20   (e.g., a point mutation; the addition, deletion and/or
substitution of one or more nucleotides).

Secondary structure has been shown to be a critical
component in determining the rates of translation
efficiency of several proteins (Bettany, A.J., et al.,
25   J. Biol. Chem. 267:16531-16537, 1992; Kozak, M., J. Mol.
Biol. 235:95-110, 1994). By implication, altered rates
of translation may affect posttranslational
modifications, for example, glycosylation patterns, and,
thus, proper folding of the resulting protein leading to
30   changes in the chemistry, structure and function of the

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protein. The modified erythropoietin variant proteins
described herein are unique in that they are composed of
mutant proteins produced by alterations in 5' and 3'
untranslated (noncoding) regions of the gene.

5   The modified secretable erythropoietin proteins
described herein provide useful reagents to further
elucidate the structure/function relationship of
erythropoietin and its cellular receptor.

        Such modified secretable erythropoietin proteins
10   with altered regulating ability can also be used for
therapeutic purposes. For example, modified
erythropoietin proteins with enhanced biological
activity would be a more potent therapeutic, therefore
requiring a lower effective dose or less frequent
15   administration to an individual. Erythropoietin
proteins with decreased biological activity that still
retain their structural integrity and bind to their
cognate receptor would be useful to decrease growth and
differentiation of red blood cell precursors in certain
20   leukemias and polycythemias. Furthermore, an
erythropoietin protein that selectively triggers only
certain events within the red blood cell precursor cell
would be useful in treating various hematological
conditions.

25   Further, it is expected that modified secretable
mutant erythropoietin proteins with increased heat
stability relative to wildtype erythropoietin proteins
would have a longer plasma half-life relative to
wildtype erythropoietin proteins. Thus, such modified
30   erythropoietin proteins with increased heat stability

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can be useful therapeutically. For example, modified
secretable mutant erythropoietin proteins with increased
heat stability would be especially important in patients
with a fever and/or experiencing an increased metabolic
5   state.

The present invention also relates to methods of
modifying or altering the regulating activity of a
secretable erythropoietin protein.

This invention further relates to pharmaceutical
10   compositions comprising an effective amount of modified
secretable human recombinant erythropoietin in a
physiologically acceptable carrier.

The present invention also relates to a method of
evaluating a substance for ability to regulate growth
15   and differentiation of red blood cell progenitor cells.

BRIEF DESCRIPTION OF THE DRAWINGS
Figure 1 is a schematic diagram of the *in vitro*
mutagenesis protocol. WT = wildtype erythropoietin.

Figure 2 depicts the structure of expression vector
20   pSV-2-erythropoietin.

Figure 3 is a graphic representation of the
specific activities of nine mutant erythropoietin
proteins.

Figure 4 is a graphic representation of the results
25   of monoclonal antibody precipitation of the mutant
erythropoietin proteins.

Figure 5 is a graphic representation of the
activity of heat-denatured wildtype erythropoietin as

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measured by radioimmunoassay (■) and the Krystal
bioassay (●).

Figure 6A-6H is a graphic representation of the
activity of the 103 mutant erythropoietin proteins as
5   measured by radioimmunoassay (■) and the activity of
wildtype erythropoietin (●).

Figure 6A shows the activity of R103A. Figure 6B
shows the activity of R103D. Figure 6C shows the
activity of R103K. Figure 6D shows the activity of
10 R103E. Figure 6E shows the activity of R103N. Figure
6F shows the activity of R103Q. Figure 6G shows the
activity of R103H. Figure 6H shows the activity of
R103L.

Figure 7 is a schematic representation discribing
15 how differences in mRNA and protein structure; and
protein function can result from alterations in the 5'
and 3' UTR of a gene.

Figures 8A-C depict the nucleotide sequence of the
human erythropoietin gene (SEQ ID NO:23).

20   Figures 9A-F depict the nucleic acid sequence of
nucleotides 401-624 in the 5' untranslated region of the
EPO gene (SEQ ID NO:24)(Figure 9A) and five variant
sequences (SEQ ID NOS: 25-29)(Figures 9B-9F).

Figures 10A-10E depict the nucleic acid sequence of
25 nucleotides 2773-2972 in the 3' untranslated region of
the EPO gene (SEQ ID NO:30)(Figure 10A) and four variant
sequences in that region (SEQ ID NOS: 31-34)(Figures
10B-10E).

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DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the identification of amino acid residues of the erythropoietin polypeptide which are critical for its

5   biological activity and secretable properties. These sites have been precisely defined through oligonucleotide-directed mutagenesis and used to create mutant human recombinant erythropoietin proteins which are altered by one, or more, amino acid substitutions

10 and thus differ from wildtype erythropoietin.   The term "recombinant", as used herein, means that a host protein is derived from recombinant (e.g., eukaryotic or prokaryotic host cell) expression systems which include, for example, yeast (e.g., Saccharomyces), bacteria (such

15 as, Escherichia or Bacillus), and animal cells including insect or mammalian expression systems. Proteins expressed in most bacterial cultures will be free of glycan. Protein expressed in yeast may have a glycosylation pattern different from protein expressed

20 in mammalian cells.

As used herein, the term nucleotide sequence or nucleic acid sequence refers to a heteropolymer of deoxyribonucleotides (DNA) or ribonucleotides (RNA).

Nucleic acid sequences encoding the proteins

25 provided in this invention can be assembled from DNA, either cDNA or genomic DNA, or RNA, and short oligonucleotide linkers to provide a synthetic nucleic acid sequence which is capable of being expressed in a recombinant transcriptional unit.   Homologous

30 nucleic acids, including DNA or RNA, can be detected

and/or isolated by hybridization (e.g., under high stringency conditions or moderate stringency conditions). "Stringency conditions" for hybridization is a term of art which refers to the conditions of

5   temperature and buffer concentration which permit hybridization of a particular nucleic acid to a second nucleic acid in which the first nucleic acid may be perfectly complementary to the second, or the first and second may share some degree of complementarity which is

10 less than perfect. For example, certain high stringency conditions can be used which distinguish perfectly complementary nucleic acids from those of less complementarity. "High stringency conditions" and "moderate stringency conditions" for nucleic acid

15 hybridizations are explained in several technical protocol reference texts, for example, Ausubel, F.M., *et al.*, *"Current Protocols in Molecular Biology"* (1995), the teachings of which are hereby incorporated by reference. The exact conditions which determine the

20 stringency of hybridization depend not only on ionic strength, temperature and the concentration of destabilizing agents such as formamide, but also on factors such as the length of the nucleic acid sequence, base composition, percent mismatch between hybridizing

25 sequences and the frequency of occurrence of subsets of that sequence within other non-identical sequences. Thus, high or moderate stringency conditions could be determined for detecting the various forms of recombinant erythropoietin.

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By varying hybridization conditions from a level of stringency at which no hybridization occurs to a level at which hybridization is first observed, conditions which will allow a given sequence to hybridize (e.g.,

5   selectively) with the most similar sequences in the sample can be determined.

Exemplary conditions are described in Krause, M.H. and Aaronson, S.A., *Methods in Enzymology, 200*:546-556, 1991. Also, *"Current Protocols in Molecular Biology"*

10   (*supra*), which describes how to determine washing conditions for moderate or low stringency conditions. Washing is the step in which conditions are usually set so as to determine a minimum level of complementarity of the hybrids. Generally, starting from the lowest

15   temperature at which only homologous hybridization occurs, each °C by which the final wash temperature is reduced (holding SSC concentration constant) allows an increase by 1% in the maximum extent of mismatching among the sequences that hybridize. Generally, doubling

20   the concentration of SSC results in an increase in T_m of -17°C. Using these guidelines, the washing temperature can be determined for high, moderate or low stringency, depending on the level of mismatch sought. For example, in this invention alterations in the noncoding regions

25   of the gene (5' and 3'untranslated regions) may necessitate changes in stringency conditions from low to medium to high depending upon the number of nucleotides that are modified that differ from the condition used to detect wild type versions of the gene. Where

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appropriate the salt concentrations and temperatures
will be adjusted accordingly.

IDENTIFICATION OF AMINO ACID RESIDUES OF HUMAN
RECOMBINANT ERYTHROPOIETIN CRITICAL FOR BIOLOGICAL
5 ACTIVITY

        Previously, anti-peptide antibodies to several
hydrophilic domains of the erythropoietin molecule had
demonstrated that antibodies to amino acids 99-110
(Domain 1) and 111-129 (Domain 2) block the hormone's
10 biological activity. Binding of the antibody to a
portion of the erythropoietin molecule that participated
in receptor recognition would block such recognition,
thereby neutralizing erythropoietin's biological
activity. (Sytkowski, A. J. and Donahue, K. A., *J.*
15 *Biol. Chem.* 262:1161-1165 (1987)).

        A series of mutants across the 99-129 region was
produced by sequentially replacing three amino acids
with Glu-Phe. Mutations in amino acid residues 99-110
caused a profound structural change which inhibited
20 secretion of the mutant erythropoietin after
biosynthesis. (Chern, Y., et al., *Eur. J. Biochem.*
202:225-229 (1991)). To precisely identify the amino
acid site, or sites, critical for receptor recognition
and biological activity, amino acids 100-109 were
25 studied by alanine scanning mutagenesis, as described in
detail in Example 1.

        Briefly, human recombinant erythropoietin cDNA
(Powell, J.W., et al., *Proc. Natl. Acad. Sci.* USA
83:6465-6469 (1986)) was inserted into the Phagemid

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vector pSELECT (Promega Corp., Madison, WI) which contains two genes for antibiotic resistance. One of these genes, specific for tetracycline resistance is always functional, while the other, specific for

5   ampicillin resistance, has been inactivated. The single-stranded template for the mutagenesis reaction was prepared by growing cultures of bacteria transformed with the Phagemid and infected with a helper phage. The resulting single-stranded DNA was isolated.

10   Two oligonucleotides were annealed to this recombinant ssDNA template. The first oligonucleotide was an ampicillin repair oligo designed to convert the vector to ampicillin resistance and the second oligonucleotide was a mutagenic oligo designed to change

15   a portion of the erythropoietin cDNA sequence.

Subsequently, the mutant second strand was synthesized *in vitro* using T4 DNA polymerase and ligated. This DNA was then transformed into a repair minus strain of *E. coli* and these cells were grown in

20   the presence of ampicillin. The phagemid was then harvested and a second round of transformation was carried out and mutants were selected on ampicillin plates. This resulted in the production of a double stranded phagemid containing both the ampicillin

25   resistance gene and the mutated erythropoietin cDNA.

Figure 1 shows the region of the erythropoietin cDNA encoding amino acids 96-113 (SEQ ID NO: 2) and the corresponding wildtype erythropoietin DNA sequence encoding amino acids 96-113 (SEQ ID NO: 3). The column

30   of numbers on the left hand side of Figure 1 indicates

the amino acid substitution. The only amino acid
residue substitutions made were as indicated. The
remainder of the human recombinant erythropoietin DNA
sequence was not altered. (The remaining, unaltered
5   human recombinant DNA sequence is not shown.) Thus, for
example, 100A (SEQ ID NO: 4) indicates that amino acid
100, normally a serine residue, was replaced by alanine,
101A (SEQ ID NO: 5) indicates that glycine 101 was
replaced by alanine, and so forth (SEQ ID NOS: 6-16).

10   Some sites were mutated more than once. For
example, amino acid 103 was mutated twice. The first
mutation was the substitution of alanine for arginine
103 (SEQ ID NO: 7) and the second substitution was
aspartic acid for arginine (SEQ ID NO: 8).

15   Two double mutants were also produced, 108A/113R
(SEQ ID NO: 12) and 109A/113R (SEQ ID NO: 13). In these
two instances, amino acids 108 and 109 were each
substituted with alanine in the second mutation and the
replacement of glycine 113 with arginine was introduced.

20 The changes in nucleotide sequence in each mutagenic
oligo are indicated in Figure 1 and Table I (SEQ ID NOS:
4-22). In Table I, the underlined nucleotides are those
which differ from the wildtype erythropoietin sequence.
A silent mutation designed to introduce a restriction

25 site, Hinf I, allowing convenient initial screening for
mutated erythropoietin cDNAs, was also introduced.

        In addition, two mutants in the region of the
erythropoietin cDNA encoding amino acids 1-26 (the
amino-terminus region) were produced. In these two

30 instances, amino acid 14, normally an arginine, was

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replaced either by alanine (14A) or aspartic acid (14D).

Each mutated erythropoietin cDNA was identified by restriction analysis, using standard laboratory protocols, and its structure was confirmed by DNA

5   sequencing. The mutated erythropoietin cDNA was then inserted into the expression vector pSV-2 (Figure 2) using standard laboratory techniques. (Mulligan, R. C., et al. Nature 277:108-114 (1979); Sambrook, et al., "Molecular Cloning: A Laboratory Manual", (1989)).

10   As described in detail in Example 2, COS-7 cells were transfected with the pSV-2-erythropoietin constructs. After three days, the supernatant medium was harvested and the biological activity of the mutant erythropoietin proteins and wildtype erythropoietin was

15   measured by the Krystal bioassay (Krystal, G., Exp. Hematol. 11:649-660 (1983)). Briefly, the bioassay of Krystal measures the effect of erythropoietin on intact mouse spleen cells. Mice were treated with phenylhydrazine to stimulate production of

20   erythropoietin-responsive red blood cell progenitor cells. After treatment, the spleens were removed, intact spleen cells were carefully isolated and incubated with various amounts of wildtype erythropoietin or the mutant erythropoietin proteins

25   described herein. After an overnight incubation, ³H thymidine was added and its incorporation into cellular DNA was measured. The amount of ³H thymidine incorporation is indicative of erythropoietin-stimulated production of red blood cells via interaction of

30   erythropoietin with its cellular receptor. The

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concentration of mutant erythropoietin protein, as well
as the concentration of wildtype erythropoietin, was
quantified by competitive radioimmunoassay (Incstar,
Stillwater, MN). Specific activities were calculated as
5   international units measured in the Krystal bioassay
divided by micrograms as measured as immunoprecipitable
protein by RIA. Both assays used wildtype recombinant
human erythropoietin standardized against the World
Health Organization Second International Reference
10   Standard preparation.

Two sets of experiments were performed in order to
determine the specific biological activities of these
mutant erythropoietin proteins. Specific activities of
nine of the mutant erythropoietin proteins (SEQ ID NOS:
15   4-13) assayed in the first set of experiments are shown
in Figure 3. As shown in Figure 3, the specific
activities are presented as a percent of the wildtype
erythropoietin activity for each mutant erythropoietin.
The amino acid replaced by alanine is indicated along
20   the horizontal axis. Table I also shows the specific
activities of the nine mutant erythropoietin proteins
(SEQ ID NOS: 4-13) as well as nine additional mutant
erythropoietin proteins (SEQ ID NOS: 14-22) again
assayed in the first set of experiments. The specific
25   activity noted in Table I is also that activity relative
to wildtype erythropoietin's activity, which is set at
100%.

As shown in Table I, substitution of alanine for
serine 104 decreased activity to approximately 16% of
30   wildtype erythropoietin (SEQ ID NO: 14). Substitution

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of alanine for leucine 105 (SEQ ID NO: 9) reduced the activity to approximately 44 percent of wildtype erythropoietin. Substitution of alanine for leucine 108 (SEQ ID NO: 15) reduced the activity to approximately

5   37% of wildtype erythropoietin.

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TABLE I

ALANINE SCANNING MUTAGENESIS OF AMINO ACIDS

100-109 OF ERYTHROPOIETIN

MUTANT	OLIGONUCLEOTIDE	SPECIFIC ACTIVITY	SEQ ID NO:
S100A	GGATAAAGCCGTCGCTGGCCTTCGCAGCCTCACGACTCTGCTTCGGG	107.9%	4
G101A	GCCGTCAGTGCCCTTCGCAGCCTCACGACTCTGCTTCGGG	126.8%	5
L102A	GCCGTCAGTGGCGCTCGCAGCCTCACC	93.3%	6
R103A	CGTCAGTGGCCTTGCCAGCCTCACGACTCTGCTTCGG	0.0%	7
R103D	CGTCAGTGGCCTTGACAGCCTCACGACTCTGCTTCGG	0.0%	8
L105A	GGCCTTCGCAGCGCCACGACTCTGCTTCGGG	44.0%	9
T106A	GCCTTCGCAGCCTCGCGACTCTGCTTCGGGC	76.9%	10
T107A	CGCAGCCTCACCGCTCTGCTTCGAGCTCTGCGAGCC	86.6%	11
L108A/G113R	GCCTCACCACTGCCTTCGAGCTCTGCGAGCC	77.3%	12
L109A/G113R	CCTCACCACTCTGGCTCGGGCTCTGCG	84.7%	13
S104A	GTGGCCTTCGCGCCCTCACGACTCTGCTTC	16.3%	14
L108A	CCTCACCACTGCGCTTCGAGCTCTGGGAGC	36.9%	15
L109A	CCTCACCACTCTGGCTCGGGCTCTGGG	70.2%	16
R103N	CGTCAGTGGCCTTAACAGCCTCACGACTCTGCTTCGG	0.0%	17
R103E	CGTCAGTGGCCTTGAGAGCCTCACGACTCTGCTTCGG	0.0%	18
R103Q	CGTCAGTGGCCTTCAGAGCCTCACGACTCTGCTTCGG	0.0%	19
R103H	CGTCAGTGGCCTTCACAGCCTCACGACTCTGCTTCGG	0.0%	20
R103L	CGTCAGTGGCCTTCTCAGCCTCACGACTCTGCTTCGG	0.0%	21
R103K	CGTCAGTGGCCTGAAGAGCCTCACGACTCTGCTTCGG	10.2%	22

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        To further characterize the muteins obtained by

   . substitution of the 103 arginine amino acid residue

      (SEQ ID NOS: 7, 8 and 17-22), a second set of

      experiments with COS-7 cells transfected as described

   5 in Example 2 with the pSV-2-erythropoietin mutant

      constructs encoding these muteins was performed. The

      supernatant medium was again harvested after three days

      and the biological activity of the mutant

      erythropoietin proteins was measured by the Krystal

   10 bioassay, the concentration of mutant erythropoietin

      protein was quantified by competitive radioimmunoassay

      (Incstar, Stillwater, MN) and specific activities

      (shown in Table II) were calculated as international

      units measured in the Krystal bioassay divided by

   15 micrograms as measured as immunoprecipitable protein by

      RIA.

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TABLE II

MUTAGENESIS OF AMINO ACID
Arg 103 OF ERYTHROPOIETIN

SEQ MUTANT	OLIGONUCLEOTIDE	SPECIFIC ACTIVITY	ID NO:
R103A	CGTCAGTGGCCTTGCCAGCCTCACGACTCTGCTTCGG	0.0%	7
R103D	CGTCAGTGGCCTTGACAGCCTCACGACTCTGCTTCGG	0.0%	8
R103N	CGTCAGTGGCCTTAACAGCCTCACGACTCTGCTTCGG	0.0%	17
R103E	CGTCAGTGGCCTTGAGAGCCTCACGACTCTGCTTCGG	0.0%	18
R103Q	CGTCAGTGGCCTTCAGAGCCTCACGACTCTGCTTCGG	0.0%	19
R103H	CGTCAGTGGCCTTCACAGCCTCACGACTCTGCTTCGG	1.7%	20
R103L	CGTCAGTGGCCTTCTCAGCCTCACGACTCTGCTTCGG	0.4%	21
R103K	CGTCAGTGGCCTGAAGAGCCTCACGACTCTGCTTCGG	25.0%	22

As shown in Table II, mutants having arginine 103
substituted by histidine (SEQ ID NO: 20) exhibited
decreased activity to approximately 1.7 % of wildtype
erythropoietin. Specific activity is again defined as
5   percent activity of wildtype erythropoietin activity.
Mutants having arginine 103 substituted by leucine (SEQ
ID NO: 21) exhibited decreased activity to
approximately 0.4 % of wildtype erythropoietin.
Mutants having arginine 103 substituted by lysine (SEQ
10 ID NO: 22) exhibited decreased activity to
approximately 25 % of wildtype erythropoietin compared

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to approximately 10 % of wildtype erythropoietin shown previously (compare Table I and Table II).

The results show that these three mutant erythropoietin proteins (SEQ ID NOS: 20-22) have some
5 intrinsic agonist activity (biological activity), thus indicating that the erythropoietin muteins (SEQ ID NOS: 20-22) must bind to the erythropoietin receptor. This phenomenon of weak agonist activity is commonly seen in pharmacologic blockers when tested at high enough
10 concentrations. Thus, it is reasonable to predict that equivalent quantities of these extremely low activity muteins would compete effectively with native erythropoietin and block activity.

As shown in Table II, mutants having arginine 103
15 substituted by alanine (SEQ ID NO: 7), aspartic acid (SEQ ID NO: 8), asparagine (SEQ ID NO: 17), glutamic acid (SEQ ID NO: 18), and glutamine (SEQ ID NO: 19) exhibited essentially no erythropoietin biological activity as was shown previously (Table I). The
20 results of these experiments indicate that amino acid position 103 is important for erythropoietin biological activity. Although all of these mutants were expressed and secreted into culture medium at rates equivalent to that seen for wildtype and other mutants, only very low
25 levels of biological activity were detected or, in some cases, no biological activity was detected. Methods described herein, such as the *ex vivo* bioassay of Krystal (Krystal, G., *Exp. Hematol.* 11:649-660 (1983)), which is an art-recognized bioassay used to evaluate
30 erythropoietin activity, showed that these inactive

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arginine 103 mutants are reduced in activity by at
least a 1000-fold below that of the wildtype human
recombinant erythropoietin.

Previously published studies indicated that
5 mutations in the Domain 1 region resulted in
biologically inactive muteins. (Chern, Y., et al.,
Eur. J. Biochem. 202:225-229 (1991)). Thus, modified
secretable erythropoietin proteins with mutations in
the Domain 1 region would not be expected to have
10 enhanced biological activity relative to wildtype
erythropoietin proteins. That is, making mutations in
this critical and highly conserved region of the
erythropoietin protein would not be expected to result
in the production of muteins with increased specific
15 activity relative to wildtype erythropoietin proteins.
Surprisingly, as shown in Table I, substitution of
alanine for serine 100 (SEQ ID NO: 4) and glycine 101
(SEQ ID NO: 5) increased the specific activity of these
mutant proteins.

20   To determine if the increased specific activity of
the muteins obtained by substitution of alanine for
serine 100 (S100A; SEQ ID NO: 4) and glycine 101
(G101A; SEQ ID NO: 5) was statistically significant, a
statistical analysis based on the Student-t
25 distribution for small samples was performed. The mean
values obtained were compared to that of wildtype
erythropoietin activity using the "difference between
two sample means" statistic (one-sided). The increased
specific activity of G101A over wildtype was found to
30 be statistically significant at the 0.05 level of

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significance. The increased specific activity of S100A
was not found to be statistically significant below the
0.010 level of significance.

          Additionally, mutants having arginine 14
  5 substituted by alanine (R14A) exhibited decreased
    activity to approximately 16.4 % of wildtype
    erythropoietin. Mutants having arginine 14 substituted
    by aspartic acid (R14D) exhibited decreased activity to
    approximately 3.9 % of wildtype erythropoietin.

10 STRUCTURAL INTEGRITY OF MUTANT ERYTHROPOIETIN PROTEINS
          Previously published studies indicated that
    mutations in the Domain 1 region in which a group of
    three amino acids was deleted and replaced with Glu-
    Phe, caused pronounced structural changes in the
15 molecule. (Chern, Y., et al., Eur. J. Biochem.
    202:225-229 (1991)). These structural changes were
    accompanied by lack of secretion of the mutant
    erythropoietin from the transfected COS-7 cells.
    Surprisingly, this phenomenon was not observed with the
20 more subtle mutations of the present invention. Thus,
    the mutant erythropoietin proteins described herein
    provide structurally intact (i.e., with the proper
    biological conformation) mutant erythropoietin
    proteins.
25   Assessment of the structural integrity of the
    mutated erythropoietin proteins of the instant
    invention was performed by a series of
    immunoprecipitation experiments using anti-peptide

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monoclonal antibodies to two domains of the protein, as
described in Example 3.

Briefly, the first monoclonal antibody recognizes
an epitope within amino acids 1-26 of erythropoietin.
5 The other monoclonal antibodies recognize distinct
epitopes within amino acids 99-129. It is known that a
gross change in the tertiary structure of
erythropoietin would result in an inability of one or
more of the monoclonal antibodies to recognize the
10 erythropoietin molecule. For example, it has been
demonstrated that radio-iodination of erythropoietin in
the presence of chloramine-T denatures the molecule,
resulting in loss of biological activity and
corresponding loss of recognition by monoclonal
15 antibody.

Figure 4 shows mutant erythropoietin protein
precipitated as percent of control of wildtype
erythropoietin precipitated using three monoclonal
antibodies designated across the horizontal axis, 1-26,
20 99-129α and 99-129β. The three erythropoietin proteins
examined were the wildtype erythropoietin, the 103
alanine mutant and the 103 aspartic acid mutant. As
seen on the left side of the graph, monoclonal 1-26
recognized each of the three recombinant erythropoietin
25 proteins with equal efficiency, indicating that
mutation of amino acid 103 to either alanine or
aspartic acid did not result in a gross distortion of
erythropoietin's conformation.

Similarly, as shown in the center of the graph,
30 monoclonal 99-129α also recognized the wildtype 103

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alanine mutant and 103 aspartic acid mutant with no
statistically significant difference among them. This
indicates that the conformation within the amino acids
99-129 is similar among the three recombinant
5 erythropoietin proteins.

Lastly, as shown on the right side of the graph,
monoclonal 99-129β recognized both mutant
erythropoietin proteins with approximately half the
efficiency as it recognized the wildtype
10 erythropoietin. This is consistent with the subtle
structural change introduced by a single amino acid
mutation. Taken together, it is reasonable to assume
that the inactive point mutants, 103 alanine and 103
aspartic acid, are not grossly denatured.

15 HEAT STABILITY OF MUTANT ERYTHROPOIETIN PROTEINS

A previously published study indicated that
recombinant human erythropoietin aggregates as
temperature rises. (Endo, Y., et al., J. Biochem.
112(5):700-706 (1992)). Most of the erythropoietin
20 molecules within these multimeric aggregates (twenty
erythropoietin molecules per aggregate) would almost
certainly not be detectable by antibodies in a
radioimmunoassay (RIA). Surprisingly, heat reduced the
RIA detection of wildtype erythropoietin much more
25 rapidly than the more stable mutants of the present
invention. Thus, some of the mutant erythropoietin
proteins described herein demonstrate increased heat
stability relative to the wildtype erythropoietin
protein.

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Assessment of the heat stability of the mutated
erythropoietin proteins of the instant invention was
performed by comparing *in vitro* biological activity
with antibody reactivity. Briefly, aliquots of
5 conditioned medium from erythropoietin cDNA-transfected
COS cells were incubated at 56°C for specified time
intervals. The samples were cooled on ice and a
fraction of each was assessed for biological activity
in the Krystal bioassay. The remainder was split into
10 two fractions and erythropoietin protein was quantified
by radioimmunoassay using the commercially available
INCSTAR RIA kit. The results are given in terms of the
percent biological activity remaining or percent
protein immunoprecipitated after heat treatment
15 compared to untreated samples.

Wildtype erythropoietin exhibits a time-dependent
decrease in biological activity when incubated at 56°C
or above (Figure 5); Tsuda, E., *et al.*, *Eur. J.
Biochem.* 188:405-411 (1990). Interestingly, a
20 corresponding decrease in the ability of the commercial
radioimmunoassay's antibodies to recognize this heat-
denatured erythropoietin was also observed (Figure 5).
This observation was quite reproducible and enabled the
use of the RIA to measure the heat stability of the
25 inactive R103A erythropoietin compared to that of
wildtype erythropoietin. As seen in Figure 6A, the
heat denaturation curves of R103A and wildtype
erythropoietin are essentially identical.

To confirm that this heat stability comparison is
30 sensitive to mutations in this region of

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erythropoietin, the effect of the aspartic acid substitution (R103D) on the protein's stability was evaluated. The introduction of a negatively charged amino acid residue would reasonably be more

5 structurally disruptive to the molecule than an alanine, and thus be more likely to alter the protein's heat-denaturation curve. The heat stability of R103D was markedly different (i.e., greater) than that of wildtype erythropoietin and R103A, as anticipated

10 (Figure 6B).

To further characterize the nature of the interaction between amino acid residue 103 and the erythropoietin receptor, site-directed mutagenesis was used to produce erythropoietin analogs with altered

15 side chain properties at this position. Arginine was substituted with histidine (R103H), lysine (R103K), asparagine (R103N), glutamine (R103Q), leucine (R103L) and glutamic acid (R103E) to generate 6 new altered erythropoietin molecules. Culture supernatants of

20 cells transfected with these constructs in a first set of experiments were tested in the Krystal bioassay and the heat stability assay for biological activity and structural stability, respectively.

The heat denaturation curve of R103K was

25 essentially identical to that generated for the wildtype protein. Interestingly, the heat denaturation curve for R103E was notably different from that of wildtype, and very similar to that of R103D. The other 4 mutants had denaturation kinetics intermediate to

30 that of these two proteins. (See Figures 6C-6H).

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PRODUCTION OF ADDITIONAL ERYTHROPOIETIN PROTEINS HAVING
ALTERED BIOLOGICAL ACTIVITY

    As a result of the identification of sites which
are critical to erythropoietin activity in terms of the
5 amino acid residue present and which can be altered to
produce a mutated sequence which has altered biological
activity but retains its structural integrity, it is
now possible to produce modified secretable human
recombinant erythropoietin proteins whose ability to
10 regulate the growth and differentiation of red blood
cell progenitors is altered (i.e., whose ability to
regulate red blood cell progenitors is different from
that of the corresponding wildtype human recombinant
erythropoietin). These modified human recombinant
15 erythropoietin proteins can be secreted in homologous
or heterologous expression systems.

    As described in the previous sections and in the
Examples, such sites have been identified by
oligonucleotide-directed mutagenesis and used to create
20 mutant erythropoietin which resulted in substitution of
amino acids at positions 100-109 within Domain 1 (SEQ
ID NO: 1), as represented in Figure 1 (SEQ ID NOS: 4-
13) and Table I (SEQ ID NOS: 4-16). The data indicate
that arginine 103 is critical for erythropoietin's
25 biological activity. Additionally, serine 104, leucine
105 and leucine 108 appear to play a role, as indicated
by the decreased biological activity of these mutants
as measured in the above-described bioassays.

    It is important to note that the ability of
30 erythropoietin to regulate growth and differentiation

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of red blood cell progenitors depends on the ability of erythropoietin to bind to its cellular receptor. Importantly, the mutations described herein do not disrupt the structural integrity of the erythropoietin

5 protein, as evidenced by the fact that the mutated protein is secreted. That is, as the data presented herein indicates, these mutant erythropoietin proteins retain their biological conformation. These results also indicate that Domain 1 amino acids 99-110 very

10 likely participate in receptor recognition and activation.

Moreover, as the data presented herein indicates, some mutant erythropoietin proteins also demonstrate increased heat stability relative to the wildtype

15 erythropoietin, even though the biological activity of the mutant has been significantly decreased.

Substitution of alanine at arginine 103 produced erythropoietin mutants with no detectable erythropoietin activity as measured by standard

20 techniques. Mutations at serine 104, leucine 105 and leucine 108 also significantly decreased biological activity relative to wildtype erythropoietin activity. In a similar manner, other changes at one or more of these critical sites can result in reduction of

25 erythropoietin activity. Conversely, amino acid residues can be introduced at these critical sites to produce modified secretable human recombinant erythropoietin proteins with enhanced biological activity relative to wildtype erythropoietin activity.

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Conservative substitutions can be made at one or
more of the amino acid sites within residues 100-109 of
the molecule. For example, alanine and aspartic acid
have been used to replace arginine 103. Substitution
5 of these amino acids by other amino acids of the same
type (i.e., a positively charged, or basic, amino acid
for a positively charged, or basic, one, or an acidic
amino acid for an acidic one) as that present at that
specific position can be made and the effect on
10 erythropoietin's ability to regulate the growth and
differentiation of red blood cell progenitors can be
determined, using the methods described herein.

Substitutions at these critical sites, alone or in
combination, of amino acids having characteristics
15 different from those of amino acids whose presence at
those sites has been shown to eliminate or reduce
erythropoietin activity can also be made and their
effect on activity assessed as described above. In
particular, substitutions of some, or all, of the amino
20 acids at one, or more, of these critical sites which
result in modified secretable erythropoietin proteins
with enhanced erythropoietin activity can be made.
Using the techniques described herein, erythropoietin
proteins having enhanced biological activity can be
25 identified.

In addition, more radical substitutions can be
made. For example, an amino acid unlike the residue
present in the corresponding position in the wildtype
sequence is substituted for the residue in wildtype
30 erythropoietin (e.g., a basic amino acid is substituted

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for an acidic amino acid). Each resulting mutant is
then evaluated using the anti-erythropoietin
immunoprecipitation techniques and biological activity
assays as described.

5   As a result, modified secretable human recombinant
erythropoietin proteins having enhanced erythropoietin
activity or increased heat stability can be identified.
Similar techniques can be used to identify additional
critical sites and subsequently, to make substitutions

10 and evaluate their effects on erythropoietin regulating
activity.

The present invention also relates to modified
erythropoietin variant mutant proteins encoded by
nucleic acids that contain alterations in noncoding

15 regions of the gene in addition to mutations in coding
regions as described above.

The variant nucleic acid molecules encoding, for
example, modified erythropoietin variant mutant
proteins created by altering the 3' and/or 5' UTR of

20 the erythropoietin gene, would preferably contain
regulatory sequences. Regulatory sequences include all
cis-acting elements that control transcription and
regulation such as, promoter sequences, enhancers,
ribosomal binding sites, and transcription binding

25 sites. Selection of the promoter will generally depend
upon the desired route for expressing the protein. For
example, where the mutein erthropoietin variant protein
is to be expressed in a recombinant eukaryotic or
prokaryotic cell, the selected promoter is recognized

30 by the host cell. A suitable promoter which can be

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used can include the native promoter for the binding
moiety which appears first in the construct.

The elements which comprise the nucleic acid
molecule can be isolated from nature, modified from
5 native sequences or manufactured *de novo*, as described,
for example, in the several art-recognized laboratory
technical protocol texts such as Sambrook, *et al.*,
"Molecular Cloning: A Laboratory Manual," (1989) and
Ausubel, *et al.* "Current Protocols in Molecular
10 Biology," (1995). The elements can then be isolated
and fused together by methods known in the art, such as
exploiting and manufacturing compatible cloning or
restriction sites.

The nucleic acid molecules encoding modified
15 erythropoietin variant proteins can be inserted into a
construct which can, optionally, replicate and/or
integrate into a recombinant host cell, by known
methods which may vary depending upon the form of the
recombinant erythropoietin mutein which is expressed.
20 The host cell can be a eukaryotic or prokaryotic cell
and includes, for example, pichia expression systems,
yeast (such as, Saccharomyces), bacteria (such as,
Escherichia or Bacillus), animal cells or tissue,
including insect (such as, *Spodoptera frugiperda 9)* or
25 mammalian cells (such as, somatic or embryonic human
cells, Chinese hamster ovary cells, HeLa cells, human
293 cells, monkey kidney COS-7 cells, baby hamster
kidney BHK cells, C127 cells, etc.). The selection of
the host cell governs the posttranslational
30 modifications that may occur. For instance,

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glycoproteins could be expressed in mammalian, insect, or yeast cells whereas nonglycosylated protein could be expressed in bacteria.

In addition, the selection of the appropriate host
5 cell may differ when expressing recombinant modified erythropoietin variant proteins manufactured by alterations in the noncoding regions of the gene. (Schultz, et al., J. Virol. 70:1041-1049, 1996).

The nucleic acid molecule can be incorporated or
10 inserted into the host cell by known methods. Examples of suitable methods of transfecting or transforming cells include calcium phosphate precipitation, electroporation, microinjection, infection, lipofection and direct uptake. Methods for preparing such
15 recombinant host cells are described in more detail in several technical books, for example, Sambrook, et al., (supra) and Ausubel, et al. (supra).

The host cells are maintained under suitable conditions for expressing and recovering the
20 recombinant modified erythropoietin variant protein. Generally, the cells are maintained in a suitable buffer and/or growth medium or nutrient source for growth of the cells and expression of the gene product(s). The growth media are generally known in
25 the art and include sources of carbon, nitrogen and sulfur. Examples include Dulbeccos modified Eagles media (DMEM), RPMI-1640, M199 and Grace's insect media. The selection of a buffer is not critical to the invention. The pH which can be selected is generally

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one tolerated by or optimal for growth for the host
cell.

The cell is maintained under a suitable
temperature. and atmosphere. For example, an aerobic
5 host cell is maintained under aerobic atmospheric
conditions or other suitable conditions for growth.
The temperature should also be selected so that the
host cell tolerates the process and can be, for
example, between about 27°C and 40°C.

10 APPLICATIONS OF MODIFIED SECRETABLE ERYTHROPOIETIN
PROTEINS HAVING ALTERED BIOLOGICAL ACTIVITY

As described above, arginine 103 is essential for
erythropoietin's biological activity. Additionally,
serine 104, leucine 105 and leucine 108 also appear to
15 play a significant role in biological activity.
Furthermore, these subtle point mutations do not
compromise the structural integrity, (i.e.,
secretability) of the erythropoietin molecule. Since
these described muteins have some intrinsic biological
20 activity as detected by the assays described herein,
albeit significantly reduced from wildtype
erythropoietin, it is reasonable to assume that they do
bind to the erythropoietin receptor. Thus, it is
reasonable to assume that the mutant erythropoietin
25 proteins will be recognized by the erythropoietin
cellular receptor in essentially the same manner as the
wildtype erythropoietin.

Modified secretable human recombinant
erythropoietin proteins of the present invention can be

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used for therapy and diagnosis of various hematologic
conditions. For example, an effective amount of
modified secretable recombinant erythropoietin with
enhanced biological activity to regulate the growth and
5 differentiation of red blood cell progenitors can be
used therapeutically (in vivo) to treat individuals who
are anemic (e.g. as a result of renal disease,
chemotherapy, radiation therapy, or AIDS). An
effective amount of modified secretable human
10 recombinant erythropoietin protein, as defined herein,
is that amount of modified secretable erythropoietin
protein sufficient to regulate growth and
differentiation of red blood cell progenitor cells.
For example, modified secretable erythropoietin protein
15 with increased regulatory ability will bind to the
erythropoietin receptor and stimulate the growth and
differentiation of red blood cell progenitor cells.
The modified secretable erythropoietin with enhanced
biological activity would be more potent than the
20 wildtype erythropoietin. Thus, to increase red blood
cell growth and differentiation in anemic conditions, a
lower effective dose or less frequent administration to
the individual would be required.

Modified secretable erythropoietin with altered
25 regulating activity can also be used to selectively
trigger only certain events regarding the growth and
differentiation of red blood cell precursors. For
example, it has recently been shown that binding of
erythropoietin to its receptor generates two distinct
30 chemical signals in cells, a protein kinase C dependent

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activation of the proto-oncogene c-myc and a
phosphatase mediated signal to c-myb. (Spangler, R.,
et al., J. Biol. Chem. 266:681-684 (1991); Patel, H. R.
and Sytkowski, A. J., Abstract 1208, Blood 78(10)
5 Suppl. 1 (1991)). Thus, a modified secretable
erythropoietin can be used to selectively activate
either the protein kinase C or the phosphatase
pathways.

An effective amount of modified secretable
10 erythropoietin with decreased biological activity
relative to wildtype erythropoietin activity, (i.e.,
reduced biological activity or no detectable biological
activity), can be used to treat individuals with
various erythroleukemias. In this case, an effective
15 amount of modified secretable erythropoietin protein
with decreased regulatory ability will bind to the
erythropoietin cellular receptor. However, upon the
mutant erythropoietin protein binding to the receptor,
it is reasonable to predict that the mutant protein
20 lacks ability to trigger subsequent erythropoietin
events. It is further reasonable to predict that,
because the mutant erythropoietin does bind to the
receptor, it prevents wildtype erythropoietin from
binding to the receptor (i.e., competitively inhibits
25 the binding of wildtype erythropoietin). Thus, the red
blood cell progenitors do not proliferate and/or
differentiate.

The mutant erythropoietin proteins of the present
invention are secretable, indicating that they retain
30 their structural integrity, and thus fully participate

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in receptor recognition and binding. The initial
interaction of a hormone with its cognate receptor
might be expected to result in further conformational
changes of the hormone ligand, thereby stabilizing the
5 hormone/receptor complex and allowing the formation of
higher ordered complexes. However, if a modified
erythropoietin protein of the present invention, with
no detectable erythropoietin activity, binds to its
receptor, it is reasonable to assume that the
10 subsequent events triggered by receptor binding will be
altered or inhibited. Therefore, it is also reasonable
to assume that growth and differentiation of red blood
cell progenitor cells will be altered or inhibited,
thereby inducing a remission in a red blood cell
15 leukemia.

Recently, a constitutively active (hormone
independent) form of the murine erythropoietin receptor
was isolated. (Watowich, S. S., *Proc. Natl. Acad. Sci.
USA* 89:2140-2144 (1992)). It has also been shown that
20 the envelope glycoproteins of certain murine viruses
bind to and activate the murine erythropoietin
receptor. (Yoshimura, A., *Proc. Natl. Acad. Sci. USA*
87:4139-4143 (1990)). Thus, erythropoietin-independent
activation (constitutive activation) of the
25 erythropoietin receptor resulting in red blood cell
proliferation in a mammal has been demonstrated. It is
reasonable to predict that similar constitutive
activation would occur in humans, (for example, a virus
similar to Rauscher or Friend virus) may constitutively
30 activate the human erythropoietin receptor also

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resulting in proliferation of red blood cell
progenitors. A modified secretable erythropoietin,
which retains its structural integrity to bind to the
receptor, yet does not activate red blood cell

5 proliferation, would be useful as an antagonist to
block such constitutive activation. Moreover, modified
secretable erythropoietin proteins with increased
stability would provide long-acting erythropoietin
antagonists.

10   Modified secretable erythropoietin would be useful
to treat other various medical disorders. For example,
polycythemia vera is characterized by uncontrollable
proliferation of red blood cells and is currently
treated by chemotherapy, radiation or phlebotomy. The

15 increased number of red blood cells increases blood
viscosity, leading to a hypertensive condition that can
result in a stroke. It is reasonable to predict that
an antagonist of erythropoietin, which binds to the
receptor and blocks activation, would be a useful, non-

20 invasive treatment.

Likewise, individuals with cyanotic heart disease
often have a hyper-erythropoietin condition, leading to
increased erythrocyte proliferation. Also, renal
disease patients that are being treated with wildtype

25 erythropoietin may experience an overdose. Once the
wildtype erythropoietin has been administered, it
continues to act. Thus, in these cases, it would be
useful to administer a modified secretable
erythropoietin with decreased activity to block the

30 effects of the endogenous and exogenous erythropoietin.

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Furthermore, certain hemolytic anemias, such as
sickle cell anemia and thalassemia, result in rapid
destruction of red blood cells. The body responds by
increasing the levels of erythropoietin produced to
5 stimulate red blood cell production. However, the red
blood cells produced carry defective hemoglobin. It
would be useful to use a modified secretable
erythropoietin to reduce production of defective
erythrocytes while another form of therapy is used to
10 stimulate normal hemoglobin synthesis.

Erythropoietin has a relatively short plasma half-
life (Spivak, J.L. and Hogans, B.B., *Blood* 73: 90-99
(1989); McMahon, F.G., *et al.*, *Blood* 76: 1718-1722
(1990)), therefore, therapeutic plasma levels are
15 rapidly lost, and repeated intravenous administrations
must be made. Although the mechanisms responsible for
this relatively short plasma half-life are not well
understood, inactivation due to heat
denaturation/aggregation is likely to play a role. A
20 previously published study indicated that
erythropoietin in human serum is susceptible to
inactivation by heat. (Elder, G.E., *et al.*, *Blood
Cells* 11: 409-419 (1986)). Thus, it is reasonable to
predict that modified secretable erythropoietin with
25 increased heat stability relative to wildtype
erythropoietin would have a longer plasma half-life
relative to wildtype erythropoietin and thus, be useful
therapeutically. This may be especially important in
patients with a fever and/or an increased metabolic
30 state.

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It is also reasonable to predict that modified secretable erythropoietins with enhanced biological activity relative to wildtype erythropoietin would require a smaller quantity relative to wildtype

5 erythropoietin to achieve a specified level of biological activity. This enhanced biological activity indicates that an effective amount of modified erythropoietin with enhanced biological activity is substantially less than a comparable effective amount

10 of wildtype erythropoietin. The effective amount of modified erythropoietin with enhanced biological activity is defined herein as the amount of modified erythropoietin required to elicit an erythropoietin response, as indicated by increased growth and/or

15 differentiation of erythrocytic precursor cells. Further, the effective amount of modified erythropoietin with enhanced biological activity will require less frequent administration than an equivalent amount of wildtype erythropoietin. For example, if an

20 effective dose of erythropoietin is typically administered three times a week, modified erythropoietin with enhanced biological activity will only need to be administered once a week. Thus, a reduced quantity of modified secretable erythropoietin

25 with enhanced biological activity would be necessary over the course of treatment than would be necessary if wildtype erythropoietin were used.

Modified secretable erythropoietin may be administered to individuals parenterally or orally.

30 The modified secretable erythropoietin proteins of this

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invention can be employed in admixture with
conventional pharmaceutically acceptable carriers.
Suitable pharmaceutical carriers include, but are not
limited to, water, salt solutions and other
5 physiologically compatible solutions. The modified
secretable erythropoietin proteins of the present
invention may be administered alone, or combined with
other therapeutic agents.

It will be appreciated that the amount of modified
10 secretable erythropoietin administered to an individual
in a specific case will vary according to the specific
modified secretable erythropoietin protein being
utilized, the particular compositions formulated, and
the mode of application. Dosages for a given
15 individual can be determined using conventional
considerations such as the severity of the condition,
body weight, age and overall health of the individual.

Modified secretable erythropoietin can also be
used for diagnostic purposes. For example, it can be
20 used in assay procedures for detecting the presence and
determining the quantity, if desired, of erythropoietin
receptor. A modified secretable erythropoietin with
enhanced activity would be useful to increase the
sensitivity and decrease the incubation times of such
25 assays. It can also be used in *in vitro* binding assays
to determine the effect of new drugs on the binding of
erythropoietin protein to its receptor.

Modified secretable erythropoietin proteins
described herein also provide useful research reagents
30 to further elucidate the role of erythropoietin in

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erythropoiesis, as well as the structure/function
relationship of erythropoietin and its cellular
receptor. For example, modified secretable
erythropoietin proteins may be useful for evaluating a
5 substance for ability to regulate growth and
differentiation of red blood cell progenitor cells. A
reasonable indication of the ability of a substance to
regulate growth and differentiation of red blood cell
progenitor cells is the extent of binding of the
10 substance to the erythropoietin receptor. The term,
extent of binding, as used herein, is defined to mean
the amount of substance bound to the receptor (e.g.,
the percent of substance bound to the receptor as
compared to a control substance that binds at
15 approximately 100 percent, or alternately, the specific
activity of the test substance). A method for
evaluating a substance for ability to regulate growth
and differentiation of red blood cell progenitor cells
can comprise comparing the extent of binding to the
20 erythropoietin receptor of the substance to be
evaluated with the extent of binding to the
erythropoietin receptor of a modified secretable mutant
erythropoietin protein. If the extent of binding to
the erythropoietin receptor of the test substance
25 (i.e., the substance to be evaluated) is comparable to
the extent of binding to the erythropoietin receptor of
the modified secretable mutant erythropoietin protein,
then the extent of binding of the test substance is an
indication that the ability of the substance to
30 regulate growth and differentiation of red blood cell

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progenitor cells is of approximately the same ability

as the modified secretable mutant erythropoietin. For

example, if the specific activity of a test peptide is

25.0%, it is reasonable to assume that the test peptide

5   has the ability to regulate growth and differentiation

of red blood cell progenitor cell comparable to the

R103K modified erythropoietin.

    The term substance, as used herein, is defined to

include proteins, e.g., analogues of wildtype

10 erythropoietin, erythropoietin protein fragments, other

proteins or peptides, and drugs.

    The extent of binding to the erythropoietin

receptor can be determined by using any of a number of

methods familiar to those of skill in the art. For

15 example, methods such as those described in Yonekura,

S. *et al.*, *Proc. Natl. Acad. Sci. USA* 88:1-5 (1991);

Chern, Y. *et al.*, *Blood* 76(11):2204-2209 (1990); and

Krystal, G., *Exp. Hematol.* 11:649-660 (1983), the

teachings of which are incorporated herein by

20 reference, may be used.

    The modified erythropoietin mutant proteins of the

invention produced, for example, by altering the 5'

and/or 3' UTR, can be used as therapeutics for delivery

to individuals having diseases or conditions that are

25 associated with deficiencies or abnormalties of the

proteins described herein. The retention and/or

deletion of nucleotides in the UTR of the

erythropoietin gene can produce heterologous

therapeutic proteins. Heterologous proteins are herein

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defined as proteins which do not exist in nature and exhibit a range of therapeutic effects.

Recombinant erythropoietin proteins with therapeutic value are known in the art. Examples

5 include Lin (U.S. Patent No. 4,703,008); Sytkowski and Grodberg (U.S. Patent No. 5,614,184); Sytkowski (U.S. Patent No. 5,580,853); and Powell (U.S. Patent No. 5,688,679):the contents of which are incorporated herein by reference.

10   For example, the modified erythropoietin proteins described herein can be employed in any method where EPO would be effective, and in particular in methods where other man-made erythropoietin proteins have not produced any clinically beneficial effect (e.g.,

15 increasing red blood cells in an anemic patient). The mode of erythropoietin administration to patients is preferably at the location of the target cells. As such, the administration can be by injection. Other modes of administration (parenteral, mucosal, systemic,

20 implant, intraperitoneal, etc.) are generally known in the art and, for erythropoietin, can be determined, for example, as described in U.S. Patent No. 5,614,184. The recombinant erythropoietin proteins can, preferably, be administered in a pharmaceutically

25 acceptable carrier, such as saline, sterile water, Ringer's solution, and isotonic sodium chloride solution.

The activity of modified erythropoietin proteins, including variants produced by alterations in the 5'

30 and/or 3' UTR, can be tested, for example, in

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pharmacological differences. Accordingly, the activity

of modified erythropoietin proteins can be evaluated

therapeutically. For example, pharmacological

differences in the secreted and purified erythropoietin

5 manufactured by the disclosed method compared to other

man-made or naturally occurring erythropoietins can

include:

1.   An increase or decrease in the potency when

administered to patients in human clinical

10   trials. The difference can be in the

required initial dose as well as maintenance

doses. A relative potency factor can be

evaluated for the modified erythropoietin

proteins.

15   2.   A reduction or increase in potential side

effects in patients may reflect altered

activities of the modified erythropoietin

proteins. For example, differences can be

manifested as an increase or decrease in

20   blood pressure which can be of extraordinary

significance in designing treatment regimens

for certain high risk patients like dialysis

patients who are, in any case, severely ill.

3.   A difference in the time lag between the

25   effect of increasing red blood cells in the

patient's serum after administration of the

modified erythropoietin proteins. This time-

lag has the consequence that the desired

therapeutic effect is either accelerated or

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delayed significantly compared to other forms
of erythropoietin. A decrease in the time
lag would be a desirable therapeutic effect
by resulting in a faster benefit to the
5   patient.

4. The ability of a patient to tolerate one form
of erythropoietin and not another. If a
patient can not tolerate one form of a
modified erythropoietin mutant protein over
10   another, this noncompatibility can indicate
therapeutic differences which in turn can
reflect structural, biochemical and
biological modifications in the various forms
of the modified erythropoietin proteins.

15 5. An increase in the circulating half-life of
EPO in patients which can result in less
frequent injections or smaller doses of EPO
having to be administered. A prolonged half-
life would not only be therapeutically
20   beneficial, but also diminish health care
costs in the treatment of chronically ill
patients.

Thus, differences in the pharmaceutical
characteristics of modified erythropoietin proteins can
25 result in variations in therapeutic effects (e.g., the
production of reticulocytes and red blood cells and an
increase in hemoglobin synthesis and iron uptake).
For example, a difference in the inherent potency which
would result in lower bioloads inflicted on the

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patient's body by administering modified erythropoietin protein which leads to an absence or drastic lowering of side effects (which may endanger the patient's life or make it impossible to administer one form of

5 erythropoietin) is particularly important in high risk patients (e.g., patients with kidney disorders) who are at high risk for hypertension, myocardial infarct or stroke.

Thus, retention, deletion, point mutation or
10 substitution in the 5' and/or 3' UTR sequences of a modified erythropoietin mutein gene fragment can ultimately influence the final structure and chemistry of the protein expressed and secreted by a host cell transfected with that gene fragment. As a consequence
15 the resulting expressed modified erythropoietin mutein protein can exhibit varying biological parameters which can be assessed using bioassays and in therapeutics.

This invention will now be illustrated by the following Examples, which are not intended to be
20 limiting in any way.

EXAMPLE 1
OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS OF HUMAN
RECOMBINANT ERYTHROPOIETIN

The oligonucleotide-directed mutagenesis used to
25 prepare the modified secretable human recombinant erythropoietin proteins of the present invention was performed using the Altered Sites™ *In Vitro* Mutagenesis System (Promega Corporation of Madison, WI). The Altered Sites™ system consists of a unique mutagenesis

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vector and a simple, straightforward procedure for
selection of oligonucleotide-directed mutants. The
system is based on the use of a second mutagenic
oligonucleotide to confer antibiotic resistance to the
5 mutant DNA strand. The system employs a phagemid
vector, pSELECT™-1, which contains two genes for
antibiotic resistance. One of these genes, for
tetracycline resistance, is always functional. The
other, for ampicillin resistance, has been inactivated.
10 An oligonucleotide is provided which restores
ampicillin resistance to the mutant strand during the
mutagenesis reaction. This oligonucleotide is annealed
to the single-stranded DNA (ssDNA) template at the same
time as the mutagenic oligonucleotide and subsequent
15 synthesis and ligation of the mutant strand links the
two. The DNA is transformed into a repair minus strain
E. coli, or other suitable host, and the cells are
grown in the presence of ampicillin, yielding large
numbers of colonies. A second round of transformation
20 in JM109, or a similar host, ensures proper segregation
of mutant and wild type plasmids and results in a high
proportion of mutants.

The pSELECT-1 plasmid is a phagemid, defined as a
chimeric plasmid containing the origin of a single-
25 stranded DNA bacteriophage. This phagemid produces
ssDNA upon infection of the host cells with the helper
phage R408 or M13KO7. The vector contains a multiple
cloning site flanked by the SP6 and T7 RNA polymerase
promoters and inserted into the lacZ α-peptide.
30 Cloning of a DNA insert into the multiple cloning site

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results in inactivation of the α-peptide. When plated

on indicator plates, colonies containing recombinant

plasmids are white in a background of blue colonies.

The SP6 and T7 promoters may be used to generate high

5 specific activity RNA probes from either strand of the

insert DNA. These sites also serve as convenient

priming sites for sequencing of the insert. The

pSELECT-1 vector carriers gene sequences for both

ampicillin and tetracycline resistance. However, the

10 plasmid is ampicillin sensitive because a frameshift

was introduced into this resistance gene by removing

the Pst I site. Therefore, propagation of the plasmid

and recombinants is performed under tetracycline

selection.

15   The pSELECT-Control vector provides a convenient

white/blue positive control for mutagenesis reactions.

This vector was derived from the pSELECT-1 vector by

removing the Pst I site within the polylinker. The

resultant frameshift in the lac α-peptide inactivated

20 β-galactosidase and led to a white colony phenotype on

indicator plates. A lacZ repair oligonucleotide

(supplied with the system) may be used to introduce a

four base insertion which corrects the defect in the

lacZ gene and restores colony color to blue. The

25 fraction of blue colonies obtained is an indication of

the mutagenesis efficiency. When the lacZ repair

oligonucleotide is used in combination with the

ampicillin repair oligonucleotide to correct this

defect, 80-90% of the ampicillin resistant colonies are

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blue. When the lacZ repair oligonucleotide is used
alone, a mutagenesis efficiency of only 2-5% is seen.

The mutagenic oligonucleotide must be
complementary to the single-stranded target DNA. The
5 ssDNA produced by the pSELECT-1 phagemid is
complementary to the lacZ coding strand.

The stability of the complex between the
oligonucleotide and the template is determined by the
base composition of the oligonucleotide and the
10 conditions under which it is annealed. In general, a
17-20 base oligonucleotide with the mismatch located in
the center will be sufficient for single base
mutations. This gives 8-10 perfectly matched
nucleotides on either side of the mismatch. For
15 mutations involving two or more mismatches,
oligonucleotides of 25 bases or longer are needed to
allow for 12-15 perfectly matched nucleotides on either
side of the mismatch.

Routinely, oligonucleotides can be annealed by
20 heating to 70°C for 5 minutes followed by slow cooling
to room temperature.

DNA to be mutated is cloned into the pSELECT-1
vector using the multiple cloning sites. The vector
DNA is then transformed into competent cells of JM109,
25 or a similar host, and recombinant colonies are
selected by plating on LB plates containing 15μg/ml
tetracycline, 0.5mM IPTG, and 40μg/ml X-Gal. After
incubation for 24 hours at 37°C, colonies containing
recombinant plasmids will appear white in a background
30 of blue colonies.

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To produce single-stranded template for the
mutagenesis reaction, individual colonies containing
pSELECT-Control or recombinant pSELECT-1 phagemids are
grown and the cultures are infected with helper phage
5 as described below. The single-stranded DNA produced
is complementary to the lacZ coding strand and
complementary to the strand of the multiple cloning
site. Two helper phages R408 and M13KO7 can be used to
provide the greatest latitude in optimizing ssDNA
10 yields.

PROTOCOL

1.   Prepare an overnight culture of cells containing
      pSELECT™-1 or pSELECT™-Control phagemid DNA by
      picking individual tetracycline resistant colonies
15   from a fresh plate. Inoculate 1-2ml of TYP broth
      (Promega) containing 15μg/ml tetracycline and
      shake at 37°C.

2.   The next morning inoculate 5ml of TYP broth
      containing 15μg/ml tetracycline with 100μl of the
20   overnight culture. Shake vigorously at 37°C for
      30 minutes in a 50ml tube.

3.   Infect the culture with helper phage R408 or
      M13KO7 at an m.o.i. (multiplicity of infection) of
      10 (i.e., add 10 helper phage particles per cell).
25   For the helper phages supplied with this system,
      add 40μl. Continue shaking for 6 hours to
      overnight with vigorous agitation.

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4. Harvest the culture supernatant by pelleting the
    cells at 12,000 x g for 15 minutes. Pour the
    supernatant into a fresh tube and spin again for
    15 minutes.

5 5. Precipitate the phage by adding 0.25 volume of
    phage precipitation solution (Promega) to the
    supernatant. Leave on ice for 30 minutes, then
    centrifuge for 15 minutes at 12,000 x g.
    Thoroughly drain the supernatant.

10 6. Resuspend the pellet in 400μl of TE buffer
    (Promega) and transfer the sample to a
    microcentrifuge tube.

7. Add 0.4ml of chloroform:isoamyl alcohol (24:1) to
    lyse the phage, vortex for 1 full minute, and
15   centrifuge in a microcentrifuge (12,000 x g) for 5
    minutes. This step removes excess PEG.

8. Transfer the upper, aqueous phase (containing
    phagemid DNA) to a fresh tube, leaving the
    interface behind. Add 0.4ml of TE-saturated
20   phenol:chloroform to the aqueous phase, vortex for
    1 full minute, and centrifuge as in step 7.

9. Transfer the upper, aqueous phase to a fresh tube
    and repeat the phenol extraction as in step 8. If
    necessary, repeat this extraction several times

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until there is no visible material at the
interface.

10. Transfer the upper, aqueous phase to a fresh tube
     and add 0.5 volume (200μl) of 7.5M ammonium
5   acetate plus 2 volumes (1.2ml) of ethanol. Mix
     and leave at -20°C for 30 minutes to precipitate
     the phagemid DNA.

11. Centrifuge at 12,000 x g for 5 minutes, remove the
     supernatant, carefully rinse the pellet with 70%
10   ethanol, and centrifuge again for 2 minutes.
     Drain the tube and dry the pellet under vacuum.
     The pellet may be difficult to see.

12. Resuspend the DNA in 20μl of H$_2$O. The amount of
     DNA present can be estimated by agarose gel
15   electrophoresis of a 2μl sample.

     The mutagenesis reaction involves annealing of the
ampicillin repair oligonucleotide and the mutagenic
oligonucleotide to the ssDNA template, followed by the
synthesis of the mutant strand with T4 DNA polymerase.
20 The heteroduplex DNA is then transformed into the
repair minus E. coli strain DMH71-18 mutS or other
suitable strain. Mutants are selected by overnight
growth in the presence of ampicillin. Plasmid DNA is
the isolated and transformed into the JM109 strain, or
25 other suitable strain. Mutant, ampicillin resistant

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colonies may be screened by direct sequencing of the
plasmid DNA.

A.   ANNEALING REACTION AND MUTANT STRAND SYNTHESIS
        The amount of oligonucleotide required in this
5 reaction may vary depending on the size and amount of
the single-stranded DNA template. The ampicillin
repair oligonucleotide (27 bases long) should be used
at a 5:1 oligo:template ratio and the mutagenic
oligonucleotide should be used at a 25:1 oligo:template
10 ratio. A typical reaction may contain approximately
100ng (0.05 pmol) of ssDNA.

PROTOCOL
    1. Prepare the mutagenesis or control annealing
       reactions as described below.

15   MUTAGENESIS ANNEALING REACTION
        Recombinant pSELECT™-1 ssDNA   0.05pmol
        Ampicillin repair oligonucleotide
        (2.2ng/μl)   1μ(0.25pmol)
        Mutagenic oligonucleotide,
20   phosphorylated (see Table 1)   1.25 pmol
        10X Annealing buffer   2μl
        Sterile H$_2$O   to final volume 20μl

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CONTROL ANNEALING REACTION

pSELECT™-Control ssDNA   100ng(0.05pmol)

Ampicillin repair oligonucleotide

   (2.2ng/μl)   1μl(0.25pmol)

5   lacZ control oligonucleotide

   (10.8ng/μl)   1μl(1.25pmol)

10X Annealing buffer   2μl

Sterile H2O   to final volume 20μl

2.   Heat the annealing reaction to 70°C for 5 minutes

10   and allow it to cool slowly to room temperature

     (15-20 minutes).

3.   Place the annealing reaction on ice and add the

     following:

     10X Synthesis buffer   3μl

15   T4 DNA polymerase (10u/μl)   1μl

     T4 DNA ligase (2u/μl)   1μl

     Sterile H_2O 5μl

                              to final volume 20μl

4.   Incubate the reaction at 37°C for 90 minutes to

20   perform mutant strand synthesis and ligation.

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TABLE 1.   AMOUNT OF MUTAGENIC OLIGONUCLEOTIDE NEEDED TO
           EQUAL 1.25 PMOL.

Primer Length	ng of Primer Equal to 1.25pmol
17mer	7.0ng
20mer	8.3ng
23mer	9.5ng
26mer	10.8ng
29mer	12.0ng

B.   TRANSFORMATION INTO BMH 71-18 MUTS PROTOCOL

1.   Add 3μl of DMSO to 200μl of BMH71-18 mut S
     competent cells, mix briefly, and then add the
     entire synthesis reaction from step A.4.

2.   Let the cells sit on ice for 30 minutes.

3.   OPTIONAL: For some strains, a heat shock at 42°C
     for 1-2 minutes after the incubation on ice has
     been reported to increase transformation
     efficiency. In our experience, however, a heat
     shock does not significantly affect the efficiency
     of transforming BMH71-18 mut S.

4.   Add 4ml of LB medium and incubate at 37°C for 1
     hour to allow the cells to recover.

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5. Add ampicillin to a final concentration of
    125μg/ml and incubate at 37°C for 12-14 hours with
    shaking.

NOTE: As a control to check the synthesis reaction, 1
5 ml of the culture can be removed after the one hour
recovery step, spun down, resuspended in 50μl of LB
medium, and plated on LB plates containing 125μg/ml
ampicillin. This is a check for the presence of
ampicillin resistant transformants; a second round of
10 transformation is necessary before screening for
mutants.

C. PLASMID MINI-PREP PROCEDURE
        This procedure is used to isolate pSELECT-1 or
pSELECT-Control plasmid DNA from the overnight culture
15 of BMH 71-18 mut S (step B.5, above). A yield of 1-3μg
of plasmid DNA may be expected.

PROTOCOL
    1. Place 1.5ml of the overnight culture into a
        microcentrifuge tube and centrifuge at 12, 000 x g
20   for 1 minute. The remainder of the overnight
        culture can be stored at 4°C.

    2. Remove the medium by aspiration, leaving the
        bacterial pellet as dry as possible.

    3. Resuspend the pellet by vortexing in 100μl of ice-
25   cold miniprep lysis buffer (Promega).

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4.   Incubate for 5 minutes at room temperature.

5.   Add 200 µl of a freshly prepared solution
     containing 0.2N NaOH, 1% SDS. Mix by inversion.
     DO NOT VORTEX. Incubate for 5 minutes on ice.

5 6.   Add 150µl of ice-cold potassium acetate solution,
        pH 4.8 (Promega). Mix by inversion or gentle
        vortexing for 10 seconds. Incubate for 5 minutes
        on ice.

7.   Centrifuge at 12,000 x g for 5 minutes.

10 8.   Transfer the supernatant to a fresh tube, avoiding
         the white precipitate.

9.   Add 1 volume of TE-saturated phenol/chloroform
     (Promega). Vortex for 1 minute and centrifuge at
     12,000 x g for 5 minutes.

15 10. Transfer the upper, aqueous phase to a fresh tube
         and add 1 volume of chloroform:isoamyl alcohol
)24:1). Vortex for 1 minute and centrifuge as in
         step 9.

11. Transfer the upper, aqueous phase to a fresh tube
20   and add 2.5 volumes of 100% ethanol. Mix and
         allow to precipitate 5 minutes on dry ice.

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12. Centrifuge at 12,000 x g for 5 minutes. Rinse the
     pellet with 70% ethanol (prechilled) and dry the
     pellet under vacuum.

13. Dissolve the pellet in 50μl of sterile deionized
5   water. Add 0.5μl of 100μg/ml DNase-free RNase A
     (Promega) and incubate for 5 minutes at room
     temperature.

14. The yield of plasmid DNA can be determined by
     electrophoresis on an agarose gel.

10 D. TRANSFORMATION INTO JM109 HOST CELLS
     PROTOCOL

1.   Add 3μl of DMSO to 200μl of JM109 competent cells,
     mix briefly, and add 0.05-0.10μg of plasmid DNA
     from step C.14. Other suitable host cells may be
15   used.

2.   Let the cells sit on ice for 30 minutes.

3.   OPTIONAL: A heat shock may be performed at this
     step.

4.   Add 2ml of LB medium and incubate at 37°C for 1
20   hour to allow the cells to recover.

5.   Divide the culture into two microcentrifuge tubes
     and spin for 1 minute in a microcentrifuge.

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6.   Pour off the supernatant and resuspended the cells
     in each tube in 50μl of LB medium.

7.   Plate the cells in each tube on an LB plate
     containing 125μg/ml ampicillin and incubate at
5   37°C for 12-14 hours.

E.   ANALYSIS OF TRANSFORMANTS

     The Altered Sites mutagenesis procedure generally
produces greater than 50% mutants, so colonies may be
screened by direct sequencing. A good strategy is to
10 pick 10 colonies and start by sequencing 4 of these.
If the mutation is located within 200-300 bases of
either end of the DNA insert, the SP6 or T7 sequencing
primers may be used for convenient priming of the
sequencing reactions.

15 EXAMPLE 2
   CELL CULTURE AND TRANSFECTION
        COS-7 cells were obtained from the American Type
   Culture Collection (Rockville, MD) and maintained in
   Dulbecco's modified Eagle's medium containing 10% fetal
20 bovine serum (GIBCO). Transient expression of cDNAs
   was performed using a DEAE-Dextran protocol modified by
   0.1mM chloroquine treatment (Sussman, D.J. & Milman,
   Mol. Cell Biol. 4:1641-1645 (1984); Ausubel, F.M., *et*
   al., "*Current Protocols in Molecular Biology*" pp.921-
25 926, John Wiley and Son, New York, (1989)). 3 days
   before the transfection, COS-7 cells were plated at 2 x
   10^5/10-cm tissue culture dish. 4μg DNA were used in

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each transfection. Medium was collected 3 days after
transfection and assayed for erythropoietin activity
and protein.

EXAMPLE 3

5 IMMUNOPRECIPITATION OF ERYTHROPOIETIN

Wildtype and mutant erythropoietin contained in
supernatant medium from COS cell transfections were
diluted one- to four-fold with Dulbecco's modified
Eagle medium containing 10% fetal bovine serum. After
10 one hour incubation at 37 degrees C with a monoclonal
anti-peptide antibody to erythropoietin directed
against amino acids 1-26 or 99-129, an equal volume of
Omnisorb (Calbiochem) was added to the samples and the
suspension was incubated for one hour at 4 degrees C.
15 The Omnisorb was pelleted by centrifugation at 4000 rpm
for 30 seconds. The erythropoietin remaining in the
supernatant which was not bound by the monoclonal
antibody was measured by radioimmunoassay. The amount
of erythropoietin bound by antibody (as a percent) was
20 calculated by subtracting the amount in the supernatant
from 100%, the starting concentration.

EXAMPLE 4

MODIFIED ERYTHROPOIETIN VARIANT PROTEINS PRODUCED BY
ALTERING NONCODING REGIONS OF THE GENE

25   Typically, variants of recombinant proteins are
made by deleting, adding or substituting nucleotides
within the coding of the gene. However, it is also
possible to make variants of recombinant proteins by

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altering the noncoding regions of genes, i.e., the 5'
and 3' untranslated regions (UTR). Modifications in
the UTR of a gene, especially in the 5' sequence as
well as in the first intron, influence the regulation
5 of translation; and, thus, the expression of proteins
(Schultz, D.E., et al., J. Virol. 70:1041-1049, 1996;
Kozak, M., J. Mol. Biol. 235:95-110, 1994; Bettany,
A.J., et al., J. Biol. Chem. 267:16531-16537, 1992;
Kozak, M., J. Biol. Chem. 266:19867-19870, 1991).

10   Alterations in the non-coding sequences of the
erythropoietin gene can result in different mRNA
secondary structure (e.g., free energy of the loop and
base pairs), translation efficiency; and subsequently,
the expression, secretion and biological activity of
15 the erythropoietin. Therefore, different forms of
modified erythropoietin proteins can be manufactured as
a result of modifications in regions which flank either
the 5' or 3' side of the coding region of the
erythropoietin gene.

20   Figure 7 is a schematic representation of changes
in mRNA structure and ultimately protein structure and
function that can result when an alteration(s) is made
in the 5' and/or 3' UTR of the erythropoietin gene.
Variations in the modified erythropoietin proteins can
25 be produced as, for example, different restriction
enzyme generated fragments of genomic sequences and/or
specific nucleotide substitutions and mutations in the
5' and/or 3' UTR of the erythropoietin coding sequence.
Oligonucleotide-directed site-specific mutagenesis

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procedures as described herein can be employed to
provide the modified erythropoietin variant proteins.

Alterations in the noncoding regions of the
erythropoietin gene can affect mRNA stability, rates of
5 translation, expression from host cells, protein
processing, export from rough endoplasmic reticulum,
extent and pattern of glycosylation, secretion dynamics
and rates of export from the cell. For example, varied
glycosylation patterns can result, which, for
10 erythropoietin, are of great importance for biological
activity (Yamaguchi, K., *et al.*, *J. Biol. Chem.*
266:20434-20439, 1991). The resulting proteins can
represent chemically, structurally and biologically
distinct forms of the modified erythropoietin variant
15 proteins.

The nucleotide sequences of the modified
erythropoietin variant proteins can be confirmed by DNA
sequencing using standard experimental procedures.
Distinctive versions of genomic erythropoietin can be
20 produced by mutations in the 5' and 3' UTR and detected
by Southern blotting. Likewise, different mRNAs can be
identified by Northern blotting. Differences in
hybridization conditions, i.e., high or low
stringencies, will be an index of the diversity of the
25 DNA and mRNA. It is possible that different genomic
sequences may require different promoters (e.g., mouse
metallothionein or 3-phosphoglycerate), vectors (e.g.,
bovine papilloma virus), and/or host cells (e.g., CHO,
BHK-21 or C127 cells) to adequately express the
30 modified erythropoietin variant proteins. The

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technical methods which can be employed for the above
mentioned experimental strategies are familiar to those
of skill in the art.   For example, detailed protocols
can be found in Sambrook, *et al.*, *"Molecular Cloning:*
5 *A Laboratory Manual,"* (1989) and Ausubel, *et al.*,
"Current Protocols in Molecular Biology, "(1995);
Powell, J.S., *et al.*, *Proc. Natl. Acad. Sci. USA*
83:6465-6469, 1986; Sytkowski and Grodberg, (U.S.
Patent No. 5,614,184); Sytkowski (U.S. Patent No.
10 5,580,853); and Powell (U.S. Patent No. 5,688,679); the
teachings of which are herein incorporated by reference
in their entirety.

Mutations in the 5' and/or 3' UTR of the
erythropoietin gene can result in altered RNA
15 structure, total free energy, stability and/or rates
and efficiency of translation (Schultz, D.E., *et al.*,
J. Virol. 70:1041-1049, 1996; Kozak, M., *J. Mol. Biol.*
235:95-110, 1994; Bettany, A.J., *et al.*, *J. Biol. Chem.*
267:16531-16537, 1992; Kozak, M., *J. Biol. Chem.*
20 266:19867-19870, 1991; Purvis, I.J., *et al.*, *Nucleic
Acids Res.* 15: 7951-62, 1987).   The secondary structure
of mRNAs play an important role in the initiation and
efficiency of translation and, thus, in protein
synthesis.

25   Computer modeling using the PC/Gene® RNAFOLD
program (IntelliGenetics, Inc.) is used to predict
differences in RNA secondary structure, specifically
the total free energy, following deletion in the 5' or
3' UTR of, for example, the erythropoietin gene
30 (Figures 9-10).   The program utilizes an algorithm

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which calculates the energies of the secondary

structure of RNA. It automatically transcribes any DNA

sequence into a single stranded RNA sequence. Since

the mRNA is single stranded, it can fold back upon

5 itself due to the complementarity of bases resulting in

various "loops". Energy must be released to form a

base-paired or looped structure and the stability of

the resulting secondary structure is determined by the

amount of energy released. Therefore, if alternative

10 structures have a free energy of formation of -50

kcal/mol and -100 kcal/mol, the latter structure is

intrinsically more likely to be formed.

For example, the free energy for RNA secondary

structure for nucleotides 401-624 in the 5' UTR of the

15 erythropoietin gene is predicted to be -161.0 kcal/mol

(SEQ ID NO:24). A 50 nucleotide deletion spanning

nucleotides 501-550 results in a total free energy of

-127.2 kcal/mol (SEQ ID NO:25), whereas a 50 nucleotide

deletion at nucleotides 551-600 (SEQ ID NO:26) results

20 in an RNA structure with -118.9 kcal/mol of free energy

indicating the importance of the size of the deletion

and location in ultimately defining mRNA secondary

structure. Larger deletions, in different portions of

the 401-624 region of the 5' UTR, yield RNA structures

25 with varying predicted energy states (SEQ ID NOS:27-

29). These results are summarized in Table 2.

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TABLE 2: SEQUENCE VARIATION IN 5' UTR-
EFFECT ON mRNA FREE ENERGY

Sequence	SEQ ID NO:	Nucleotide Length (bp)	Region of Deletion	Number of Nucleotides Deleted (bp)	Free Energy (kal/mol)
Native	27	224	---	---	-161.0
5'a	25	174	501-550	50	-127.2
5'c	25	174	551-600	50	-118.9
5'c	27	124	401-550	150	-94.1
5'd	28	74	401-550	150	-52.3
5'e	29	34	401-590	190	-11.3

Likewise, for example, the free energy of RNA
secondary structure for nucleotides 2773-2972 in the
3' UTR of the eryhthropoietin gene is predicted to
be -81.4 kcal/mol (SEQ ID NO:30). A 50 nucleotide
deletion spanning nucleotides 2923-2972 (SEQ ID
NO:31) results in a total free energy of -53.5
kcal/mol, whereas a 100 nucleotide deletion at
nucleotides 2873-2972 (SEQ ID NO:32) results in an
RNA structure with -33.3 kcal/mol of free energy.
Larger deletions, in different portions of the 2773-
2973 region of the 3' UTR, yield RNA structures with
varying predicted energy states (SEQ ID NOS:33 and
34). These results are summarized in Table 3.

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TABLE 3: SEQUENCE VARIATION IN 3' UTR-
EFFECT ON mRNA FREE ENERGY

Sequence	SEQ ID NO:	Nucleotide Length (bp)	Region of Deletion	Number of Nucleotides Deleted (bp)	Free Energy (kal/ mol)
Native	30	200	---	---	-81.4
3'a	31	150	2923-2972	50	-53.5
3'e	32	100	2873-2972	100	-33.3
3'c	33	50	2823-2972	1●0	-12.5
3'd	34	100	2801-2900	100	-36.6

The secondary structure of mRNA affects the
rates of translation of the corresponding coding
regions (Kikinis, Z., et al., *Nucleic Acids Res. 23*:
4190-4195, 1995; Kozak, M., *Mamm. Genome 7*: 563-574,
1996; Bettany, A.J., et al., *J. Biol. Chem. 267*:
16531-16537, 1992; Kozak, M., *J. Mol. Biol. 235*: 95-
110, 1994). Secondary structure loops in the mRNA
must be unwound to facilitate ribosome attachment
and proper protein assembly (Alberts, B., et al.,
"Molecular Biology of the Cell" , 3rd ed., Garland
Publishing, Inc., New York, NY, pp. 223-290, 1994).
The nascent polypeptide chains can interact
with chaperon proteins, for example, BiP, in unique
ways which can affect the proper folding of the
polypeptide chain and influence passage of the
protein through the endoplasmic reticulum thereby
altering glycosylation of the resulting protein.

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Recent data suggest that BiP-like proteins not only
bind improperly folded proteins but also may assist
in the appropriate protein folding and facilitate
the membrane translocation and glycosylation of

5 secretory proteins. (Knittler, M.R., et al., *EMBO
   J.*,11:1573-1581, (1992); Sanders, S.L., et al.,
   Cell, 69:353-365, (1992)). Alterations in
   glycosylation patterns can influence the secretion
   and, in the case of erythropoietin, drastically

10 alter biological activity (Yamaguchi, K., et al., *J.
   Biol. Chem.*, 266:20434-20439, 1991).

   The three dimensional structure of
   erythropoietin is significantly influenced by the
   protein backbone and the oligosaccharide chains.

15 Alterations in the carbohydrate composition (e.g.,
   the number of N- or O-linked oligosaccharide
   residues and/or type of sugar moieties) can lead to
   different biological properties of the modified
   erythropoietin variant proteins and, thus, different

20 therapeutic effects. Therefore, an alteration in
   the 5' or 3' UTR can affect mRNA secondary
   structure, which in turn can influence the rate of
   expression and post-translational modifications such
   as glycosylation. The proper glycosylation of

25 erythropoietin is of paramount importance to proper
   folding and secretion of the mature product and,
   hence, its biological and pharmacological
   properties.

   Indices of intrinsic structural variations in

30 the modified erythropoietin proteins can be

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manifested in differences in the three-dimensional
structure of the protein backbone and the extent and
pattern of carbohydrate chains. For example,
circular dichroism (CD) spectra and thermal
5 stability for the resulting erythropoietin mutant
proteins can be performed to determine the content
of alpha helix, beta sheet, beta turn and random
coil for different glycoproteins. The structure of
the oligosaccharide chains can be determined, for
10 example, using enzymatic and chemical
deglycosylation, gas chromatography, methylation
analyses, fast-atom-bombardment mass spectrometry as
well as one-and two-dimensional ^{1}H-NMR spectrometry.
The methods to perform the above mentioned analyses
15 are routine to one of ordinary skill in the art and
are delineated in detail in several references
including for example, Ausubel, F.M., et al.,
"Current Protocols in Molecular Biology" (1995);
Nimtz, M., et al. Eur. J. Biochem. 213: 39-56, 1993;
20 and Nimtz, M., et al., FEBS 365: 203-208, 1995, the
teachings of which are herein incorporated by
reference in their entirety.

In addition, assessment of the structural
differences in the modified erythropoietin variant
25 proteins can be evaluated using immunoprecipitation
with erythropoietin-specific monoclonal antibodies
and heat denaturation curves. Experimental
techniques to measure these properties of
erythropoietin are described in Sytkowski and
30 Grodberg (U.S. Patent No. 5,614,184); Sytkowski

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(U.S. Patent No. 5,580,853); and Powell (U.S. Patent No. 5,688,679); the teachings of which are herein incorporated by reference in their entirety.

EXAMPLE 5

5 EVALUATION OF BIOLOGICAL ACTIVITY OF MODIFIED ERYTHROPOIETIN VARIANT PROTEINS

The biological activity of the modified erythropoietin variant proteins is determined using *in vitro* and *in vivo* assays.

10   The modified erythropoietin variant proteins can be preferably purified substantially prior to use, particularly where the protein could be employed as an *in vivo* therapeutic, although the degree of purity is not necessarily critical where

15 the molecule is to be used *in vitro* . The modified erythropoietin variant proteins can be isolated to about 50% purity (by weight), more preferably to about 80% by weight or about 95% by weight. It is most preferred to utilize a protein which is

20 essentially pure (e.g., about 99% by weight or to homogeneity) for *in vitro* and *in vivo* assays as well as *in vivo* therapeutics.

For example, the modified erythropoietin variant proteins, which are prepared according to

25 the methods discussed in the Examples, can be screened for *in vitro* and *in vivo* activity prior to use in therapeutic settings. The *in vitro* assay measures the effect of erythropoietin variant proteins on erythropoiesis in intact mouse spleen

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cells according to the procedure of Krystal, G.,

Exp. Hematol. 11:649-660 (1983). To screen the

various modified erythropoietin variant proteins for

activity, for example, *in vitro* or *in vivo*, the

5 proteins (or mixtures of the modified erythropoietin

variant proteins) can be evaluated for the extent of

erythropoiesis or receptor binding. Tests to

determine biological activity are well-known to

those of skill in the art. For example, the

10 biological activity of erythropoietin can be

measured as described in Sytkowski and Grodberg

(U.S. Patent No. 5,614,184); Sytkowski (U.S. Patent

Nos 5,580,853); Sytkowski, U.S. patent application

"Modified Polypeptides with Altered Biological

15 Activity", filed February 3, 1998; and Powell (U.S.

Patent No. 5,688,679); the teachings of which are

herein incorporated by reference in their entirety.

EQUIVALENTS

While this invention has been particularly

20 shown and described with references to preferred

embodiments thereof, it will be understood by those

skilled in the art that various changes in form and

details may be made therein without departing from

the spirit and scope of the invention as defined by

25 the appended claims. Those skilled in the art will

recognize or be able to ascertain using no more than

routine experimentation, many equivalents to the

specific embodiments of the invention described

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specifically herein. Such equivalents are intended
to be encompassed in the scope of the claims.

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CLAIMS

What is claimed is:

1.   An isolated nucleic acid encoding
        erythropoietin wherein the nucleic acid has one
5   or more mutations in a noncoding region, and
        wherein the erythropoietin has altered
        biological activity.

2.   The nucleic acid of Claim 1, wherein the
        mutation is in the 5' noncoding region.

10 3.   The nucleic acid of Claim 2 wherein the nucleic
        acid comprises a nucleic acid selected from the
        group consisting of SEQ ID NO:25; SEQ ID NO:26;
        SEQ ID NO:27; SEQ ID NO: 28; and SEQ ID NO: 29.

4.   A composition comprising an erythropoietin
15   protein of Claim 2 and a pharmaceutically
        acceptable carrier.

5.   A method of treating or preventing anemia in a
        mammal comprising administering to the mammal a
        therapeutically effective amount of an
20   erythropoietin protein encoded by the nucleic
        acid of Claim 2.

6.   The nucleic acid of Claim 1, wherein the
        mutation is in the 3' noncoding region.

7.   The nucleic acid of Claim 6 wherein the nucleic
      acid comprises a nucleic acid selected from the
      group consisting of SEQ ID NO: 31; SEQ ID NO:
      32; SEQ ID NO: 33; and SEQ ID NO: 34.

5 8.   A composition comprising an erythropoietin
        protein of Claim 6 and a pharmaceutically
        acceptable carrier.

9.   A method of treating or preventing anemia in a
      mammal comprising administering to the mammal a
10   therapeutically effective amount of an
      erythropoietin protein encoded by the nucleic
      acid of Claim 6.

10.   The nucleic acid of Claim 1 wherein the
      mutation is in both the 5' and 3'
15   noncoding region.

11.   The nucleic acid of Claim 10 wherein the
      nucleic acid comprises a nucleic acid selected
      from the group consisting of SEQ ID NO:25; SEQ
      ID NO:26; SEQ ID NO:27; SEQ ID NO: 28; SEQ ID
20   NO: 29; SEQ ID NO: 31; SEQ ID NO: 32; SEQ ID
      NO: 33; and SEQ ID NO: 34.

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12.   A composition comprising an erythropoietin
      protein of Claim 10 and a pharmaceutically
      acceptable carrier.

13.   A method of treating or preventing anemia in a
5   mammal comprising administering to the mammal a
      therapeutically effective amount of an
      erythropoietin protein encoded by the nucleic
      acid of Claim 10.

14.   The nucleic acid sequence of Claim 1
10   further comprising one or more mutations
      in the coding region encoding
      erythropoietin having an amino acid
      residue which differs from the amino acid
      residue present in the corresponding
15   position in wildtype erythropoietin, the
      amino acid residue of wildtype
      erythropoietin selected from the group
      consisting of amino acid 101, amino acid
      residue 103, amino acid residue 104, amino
20   acid residue 105 and amino acid residue
      108.

15.   A mutant erythropoietin protein according
      to Claim 14, wherein the amino acid
      residue at the position 101 is alanine.

25 16.   A mutant erythropoietin protein according
      to Claim 14, wherein the amino acid

residue at the position 103 is selected

from the group consisting of aspartate,

alanine, glutamate, histidine and lysine.

17.   A mutant erythropoietin protein according

5   to Claim 14, wherein the amino acid

residue at the position 104 is alanine.

18.   A mutant erythropoietin protein according

to Claim 14, wherein the amino acid

residue at the position 105 is alanine.

10 19.   A mutant erythropoietin protein according

to Claim 14, wherein the amino acid

residue at the position 108 is alanine.

ALANINE SCANNING MUTAGENESIS OF EPO 100-109
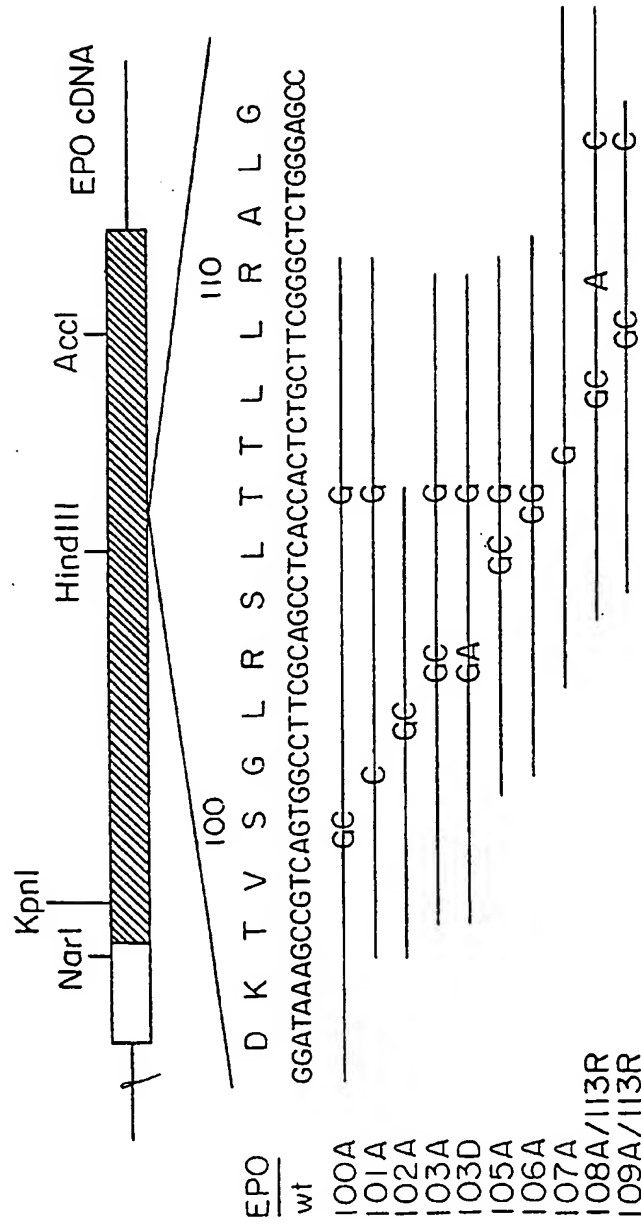
FIG. 1
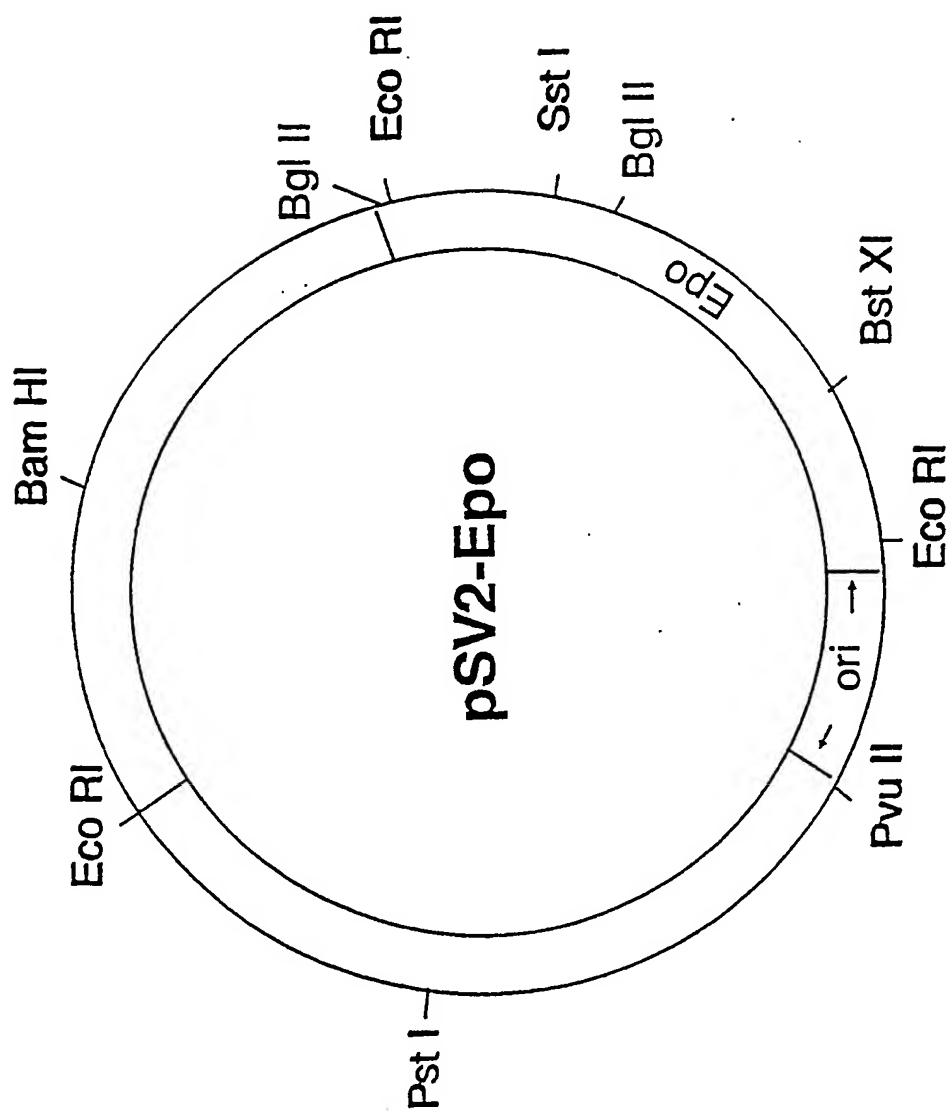
FIG. 2

ARGININE 103 IS ESSENTIAL FOR EPO'S ACTIVITY
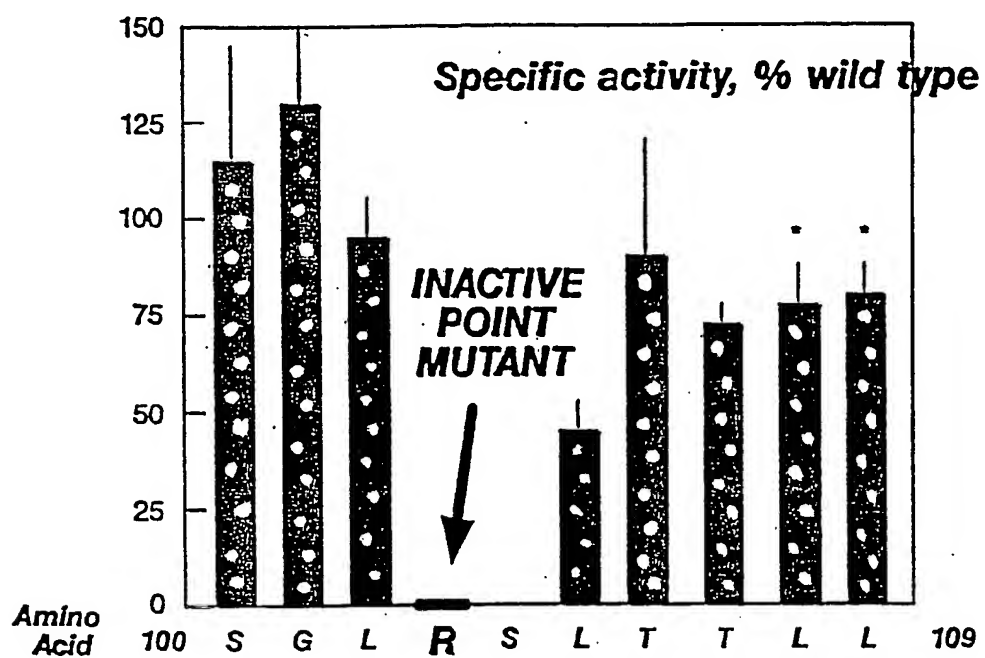
FIG. 3

MoAbs TO AMINO ACIDS 1-26 AND 99-129
PROBE THE STRUCTURE OF EPO
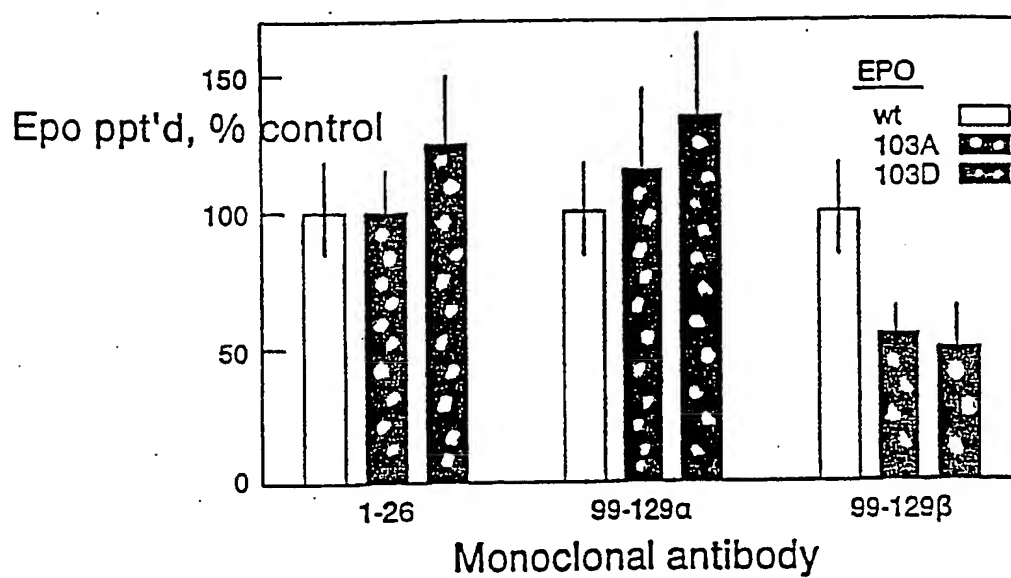
FIG. 4

FIG. 5

Epo protein ppt'd, %

Time, min.
R103A

FIG. 6A

Epo protein ppt'd, %

Time, min.
R103D

FIG. 6B

Time, min.
R103K
FIG. 6C

Time, min.
R103E

FIG. 6D

Time, min.
R103N

FIG. 6E

Time, min.
R103Q

FIG. 6F

Time, min.
R103H

FIG. 6G

Time, min.
R103L

FIG. 6H

VARIATIONS OF RECOMBINANT EPO

DNA ⟶ RNA ⟶ PROTEIN

UTR | CODING REGION | UTR

5'   3'

DNA

Modifications in Noncoding Regions

No
Modification

5' UTR
Modification

3' UTR
Modification

Different RNA Secondary Structure

Differences in:

Translation
Post-Translational
Secretion

Different Glyco protein

Different Biochemical/Structural Properties

Different Pharmacological/Therapeutic Effects
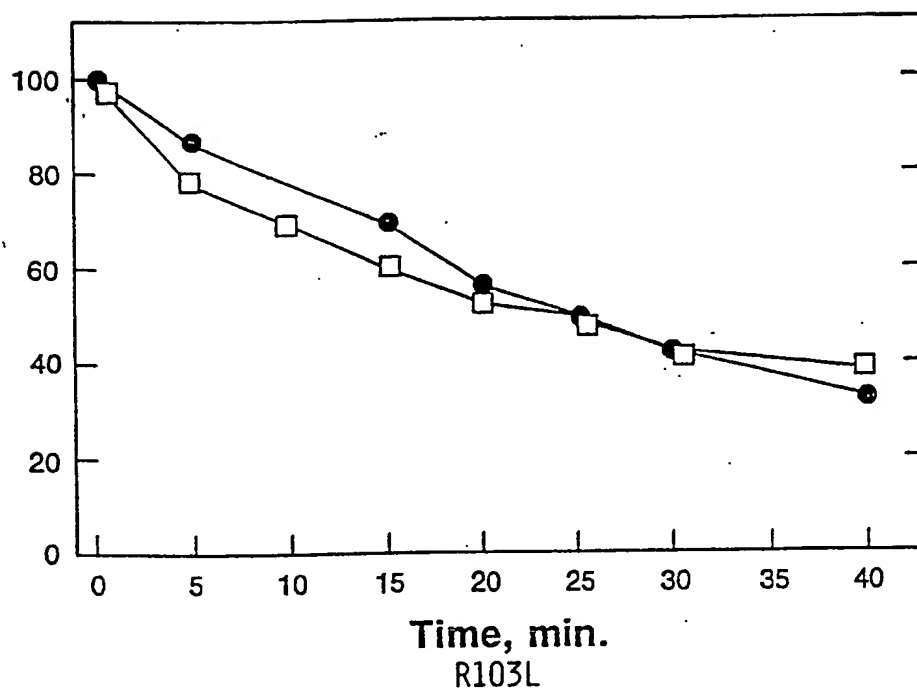
FIG. 7

HUMAN ERYTHROPOIETIN GENE

From: HOMO SAPIENS (HUMAN)
        EUKARYOTA; ANIMALIA; METAZOA; CHORDATA; VERTEBRATA; MAMMALIA;
        THERIA; EUTHERIA; PRIMATES; HAPLORHINI; CATARRHINI; HOMINIDAE.

```
1      AAGCTTCTGG GCTTCCAGAC CCAGCTACTT TGCGGAACTC AGCAACCCAG GCATCTCTGA
61     GTCTCCGCCC AAGACCGGGA TGCCCCCCAG GGGAGGTGTC CGGGAGCCCA GCCTTTCCCA
121    GATAGCACGC TCCGCCAGTC CCAAGGGTGC GCAACCGGCT GCACTCCCCT CCCGCGACCC
181    AGGGCCCGGG AGCAGCCCCC ATGACCCACA CGCACGTCTG CAGCAGCCCC GCTCACGCCC
241    CGGCGAGCCT CAACCCAGGC GTCCTGCCCC TGCTCTGACC CCGGGTGGCC CCTACCCCTG
301    GCGACCCCTC ACGCACACAG CCTCTCCCCC ACCCCACCC GCGCACGCAC ACATGCAGAT
361    AACAGCCCCG ACCCCGGCC AGAGCCGCAG AGTCCCTGGG CCACCCCGGC CGCTCGCTGC
421    GCTGCGCCGC ACCGCGCTGT CCTCCCGGAG CCGGACCGGG GCCACCGCGC CCGCTCTGCT
481    CCGACACCGC GCCCCTGGA CAGCCGCCCT CTCCTCTAGG CCCGTGGGGC TGGCCCTGCA
541    CCGCCGAGCT TCCCGGGATG AGGGCCCCCG GTGTGGTCAC CCGGCGCGCC CCAGGTCGCT
601    GAGGGACCCC GGCCAGGCGC GGAGATGGGG GTGCACGGTG AGTACTCGCG GGCTGGGCGC
661    TCCCGCCGCC CGGGTCCCTG TTTGAGCGGG GATTTAGCGC CCCGGCTATT GGCCAGGAGG
721    TGGCTGGGTT CAAGGACCGG CGACTTGTCA AGGACCCCGG AAGGGGGAGG GGGGTGGGGC
781    AGCCTCCACG TGCCAGCGGG GACTTGGGGG AGTCCTTGGG GATGGCAAAA ACCTGACCTG
841    TGAAGGGGAC ACAGTTTGGG GGTTGAGGGG AAGAAGGTTT GGGGGTTCTG CTGTGCCAGT
901    GGAGAGGAAG CTGATAAGCT GATAACCTGG GCGCTGGAGC CACCACTTAT CTGCCAGAGG
961    GGAAGCCTCT GTCACACCAG GATTGAAGTT TGGCCGGAGA AGTGGATGCT GGTAGCTGGG
1021   GGTGGGGTGT GCACACGGCA GCAGGATTGA ATGAAGGCCA GGGAGGCAGC ACCTGAGTGC
1081   TTGCATGGTT GGGGACAGGA AGGACGAGCT GGGGCAGAGA CGTGGGGATG AAGGAAGCTG
1141   TCCTTCCACA GCCACCCTTC TCCCTCCCCG CCTGACTCTC AGCCTGGCTA TCTGTTCTAG
1201   AATGTCCTGC CTGGCTGTGG CTTCTCCTGT CCCTGCTGTC GCTCCCTCTG GGCCTCCCAG
1261   TCCTGGGCGC CCCACCACGC CTCATCTGTG ACAGCCGAGT CCTGGAGAGG TACCTCTTGG
1321   AGGCCAAGGA GGCCGAGAAT ATCACGGTGA GACCCCTTCC CCAGCACATT CCACAGAACT
```

FIGURE 8A

```
1381  CACGCTCAGG GCTTCAGGGA ACTCCTCCCA GATCCAGGAA CCTGGCACTT GGTTTGGGGT
1411  GGAGTTGGGA AGCTAGACAC TGCCCCCCTA CATAAGAATA AGTCTGGTGG CCCCAAACCA
1501  TACCTGGAAA CTAGGCAAGG AGCAAAGCCA GCAGATCCTA CGGCCTGTGG GCCAGGGCCA
1561  GAGCCTTCAG GGACCCTTGA CTCCCCGGGC TGTGTGCATT TCAGACGGGC TGTGCTGAAC
1621  ACTGCAGCTT GAATGAGAAT ATCACTGTCC CAGACACCAA AGTTAATTTC TATGCCTGGA
1681  AGAGGATGGA GGTGAGTTCC TTTTTTTTTT TTTTTCCTTT CTTTTGGAGA ATCTCATTTG
1741  CGAGCCTGAT TTTGGATGAA AGGGAGAATG ATCGGGGGAA AGGTAAAATG GAGCAGCAGA
1801  GATGAGGCTG CCTGGGCGCA GAGGCTCACG TCTATAATCC CAGGCTGAGA TGGCCGAGAT
1861  GGGAGAATTG CTTGAGCCCT GGAGTTTCAG ACCAACCTAG GCAGCATAGT GAGATCCCCC
1921  ATCTCTACAA ACATTTAAAA AAATTAGTCA GGTGAAGTGG TGCATGGTGG TAGTCCCAGA
1981  TATTTGGAAG GCTGAGGCGG GAGGATCGCT TGAGCCCAGG AATTTGAGGC TGCAGTGAGC
2041  TGTGATCACA CCACTGCACT CCAGCCTCAG TGACAGAGTG AGGCCCTGTC TCAAAAAAGA
2101  AAAGAAAAAA GAAAAATAAT GAGGGCTGTA TGGAATACAT TCATTATTCA TTCACTCACT
2161  CACTCACTCA TTCATTCATT CATTCATTCA ACAAGTCTTA TTGCATACCT TCTGTTTGCT
2221  CAGCTTGGTG CTTGGGGCTG CTGAGGGGCA GGAGGGAGAG GGTGACATGG GTCAGCTGAC
2281  TCCCAGAGTC CACTCCCGTG AGGTCGGGCA GCAGGCCGTA GAAGTCTGGC AGGGCCTGGC
2341  CCTGCTGTCG GAAGCTGTCC TGCGGGGCCA GGCCCTGTTG GTCAACTCTT CCCAGCCGTG
2401  GGAGCCCCTG CAGCTGCATG TGGATAAAGC CGTCAGTGGC CTTCGCAGCC TCACCACTCT
2461  GCTTCGGGCT CTGGGAGCCC AGGTGAGTAG GAGCGGACAC TTCTGCTTGC CCTTTCTGTA
2521  AGAAGGGGAG AAGGGTCTTG CTAAGGAGTA CAGGAACTGT CCGTATTCCT TCCCTTTCTG
2581  TGGCACTGCA GCGACCTCCT GTTTTCTCCT TGGCAGAAGG AAGCCATCTC CCCTCCAGAT
2641  GCGGCCTCAG CTGCTCCACT CCGAACAATC ACTGCTGACA CTTTCCGCAA ACTCTTCCGA
2701  GTCTACTCCA ATTTCCTCCG GGGAAAGCTG AAGCTGTACA CAGGGGAGGC CTGCAGGACA
2761  GGGGACAGAT GACCAGGTGT GTCCACCTGG GCATATCCAC CACCTCCCTC ACCAACATTG
2821  CTTGTGCCAC ACCCTCCCCC GCCACTCCTG AACCCGTCG AGGGGCTCTC AGCTCAGCGC
2881  CAGCCTGTCC CATGGACACT CCAGTGCCAG CAATGACATC TCAGGGGCCA GAGGAACTGT
2941  CCAGAGAGCA ACTCTGAGAT CTAAGGATGT CACAGGGCCA ACTTGAGGGC CCAGAGCAGG
```

FIGURE 8B

```
3001  AAGCATTCAG AGAGCAGCTT TAAACTCAGG GACAGAGCCA TGCTGGGAAG ACGCCTGAGC
3061  TCACTCGGCA CCCTGCAAAA TTTGATGCCA GGACACGCTT TGGAGGCGAT TTACCTGTTT
3121  TCGCACCTAC CATCAGGGAC AGGATGACCT GGAGAACTTA GGTGGCAAGC TGTGACTTCT
3181  CCAGGTCTCA CGGGCATGGG CACTCCCTTG GTGGCAAGAG CCCCCTTGAC ACCGGGGTGG
3241  TGGGAACCAT GAAGACAGGA TGGGGGCTGG CCTCTGGCTC TCATGGGGTC CAAGTTTTGT
3301  GTATTCTTCA ACCTCATTGA CAAGAACTGA AACCACCAAT ATGACTCTTG GCTTTTCTGT
3361  TTTCTGGGAA CCTCCAAATC CCCTGGCTCT GTCCCACTCC TGGCAGCAGT GCAGCAGGTC
3421  CAGGTCCGGG AAATGAGGGG TGGAGGGGGC TGGGCCCTAC GTGCTGTCTC ACACAGCCTG
3481  TCTGACCTCT CGACCTACCG GCCTAGGCCA CAAGCTCTGC CTACGCTGGT CAATAAGGTG
3541  TCTCCATTCA AGGCCTCACC GCAGTAAGGC AGCTGCCAAC CCTGCCCAGG GCAAGGCTGC
3601  AG
```

FIGURE 8C

```
1    CCACCCCGGC CGCTCGCTGC GCTGCGCCGC ACCGCGCTGT CCTCCCGGAG CCGGACCGGG
61   GCCACCGCGC CCGCTCTGCT CCGACACCGC GCCCCCTGGA CAGCCGCCCT CTCCTCTAGG
121  CCCGTGGGGC TGGCCCTGCA CCGCCGAGCT TCCCGGGATG AGGGCCCCCG GTGTGGTCAC
181  CCGGCGCGCC CCAGGTCGCT GAGGGACCCC GGCCAGGCGC GGAG
```

Total number of bases is: 224.   FIGURE 9A

```
1    CCACCCCGGC CGCTCGCTGC GCTGCGCCGC ACCGCGCTGT CCTCCCGGAG CCGGACCGGG
61   GCCACCGCGC CCGCTCTGCT CCGACACCGC GCCCCCTGGA TCCCGGGATG AGGGCCCCCG
121  GTGTGGTCAC CCGGCGCGCC CCAGGTCGCT GAGGGACCCC GGCCAGGCGC GGAG
```

Total number of bases is: 174   FIGURE 9B

```
1    CCACCCCGGC CGCTCGCTGC GCTGCGCCGC ACCGCGCTGT CCTCCCGGAG CCGGACCGGG
61   GCCACCGCGC CCGCTCTGCT CCGACACCGC GCCCCCTGGA CAGCCGCCCT CTCCTCTAGG
121  CCCGTGGGGC TGGCCCTGCA CCGCCGAGCT GAGGGACCCC GGCCAGGCGC GGAG
```

Total number of bases is: 174   FIGURE 9C

```
1    CAGCCGCCCT CTCCTCTAGG CCCGTGGGGC TGGCCCTGCA CCGCCGAGCT TCCCGGGATG
61   AGGGCCCCCG GTGTGGTCAC CCGGCGCGCC CCAGGTCGCT GAGGGACCCC GGCCAGGCGC
121  GGAG
```

Total number of bases is: 124   FIGURE 9D

```
1    TCCCGGGATG AGGGCCCCCG GTGTGGTCAC CCGGCGCGCC CCAGGTCGCT GAGGGACCCC
61   GGCCAGGCGC GGAG
```

Total number of bases is: 74   FIGURE 9E

```
1    CCAGGTCGCT GAGGGACCCC GGCCAGGCGC GGAG
```

Total number of bases is: 34   FIGURE 9F

```
1      CCAGGTGTGT CCACCTGGGC ATATCCACCA CCTCCCTCAC CAACATTGCT TGTGCCACAC
61     CCTCCCCCGC CACTCCTGAA CCCCGTCGAG GGGCTCTCAG CTCAGCGCCA GCCTGTCCCA
121    TGGACACTCC AGTGCCAGCA ATGACATCTC AGGGGCCAGA GGAACTGTCC AGAGAGCAAC
181    TCTGAGATCT AAGGATGTCA
```

Total number of bases is: 200   FIGURE 10A

```
1      CCAGGTGTGT CCACCTGGGC ATATCCACCA CCTCCCTCAC CCACATTGCT TGTGCCACAC
61     CCTCCCCCGC CACTCCTGAA CCCCGTCGAG GGGCTCTCAG CTCAGCGCCA GCCTGTCCCA
121    TGGACACTCC AGTGCCAGCA ATGACATCTC
```

Total number of bases is: 150   FIGURE 10B

```
1      CCAGGTGTGT CCACCTGGGC ATATCCACCA CCTCCCTCAC CAACATTGCT TGTGCCACAC
61     CCTCCCCCGC CACTCCTGAA CCCCGTCGAG GGGCTCTCAG
```

Total number of bases is: 100   FIGURE 10C

```
1      CCAGGTGTGT CCACCTGGGC ATATCCACCA CCTCCCTCAC CAACATTGCT
```

Total number of bases is: 50   FIGURE 10D

```
1      CCAGGTGTGT CCACCTGGGC ATATCCACCC AGTGCCAGCA ATGACATCTC AGGGGCCAGA
61     GGAACTGTCC AGAGAGCAAC TCTGAGATCT AAGGATGTCA
```

Total number of bases is: 100   FIGURE 10E

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C07K14/505

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 02611 A (NEW ENGLAND DEACONESS HOSPITAL) 3 February 1994 see claims 1,4-8,11-14,17-23 see figure 1; table 1 ---	1-6,8-19
X	WO 95 33057 A (MENARINI RICERCHE SUD SPA ;MELE ANTONIO (IT); SANTIS RITA DE (IT);) 7 December 1995 see claims 9,10,19,20 see examples 1-3 see figure 5; table 1 --- -/--	1,2,4-6, 8,9,12, 13

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 June 1999	07/07/1999

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer van de Kamp, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	HO ET AL.: "Use of a marked erythropoietin gene for investigation of its cis-acting elements" J. BIOL. CHEM., vol. 270, no. 17, 28 April 1995, pages 10084-10090, XP002105613 see abstract see figure 1	1-3,6,7, 10,11
X	MCGARY E C ET AL: "Post-transcriptional regulation of erythropoietin mRNA stability by erythropoietin mRNA-binding protein" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 13, 28 March 1997, pages 8628-8634, XP002084635 see the whole document	1-3,6,7, 10,11
X	EP 0 409 113 A (BEHRINGWERKE AG) 23 January 1991 see claims 11,12	4,5,8,9, 12,13
A	BLANCHARD K L ET AL: "HYPOXIC INDUCTION OF THE HUMAN ERYTHROPOIETIN GENE: COOPERATION BETWEEN THE PROMOTER AND ENHANCER, EACH OF WHICH CONTAINS STEROID RECEPTOR RESPONSE ELEMENTS" MOLECULAR AND CELLULAR BIOLOGY, vol. 12, no. 12, 1 December 1992, pages 5373-5385, XP000562136 see the whole document	1-3,6,7, 10,11
P,X, L	WO 99 02710 A (BETH ISRAEL HOSPITAL ;SYTKOWSKI ARTHUR J (US)) 21 January 1999 L: PRIORITY see claims 18-33 see example 7	1-14,16

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Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority. namely:
   > Remark: Although claims 5.9 and 13
   > are directed to a method of treatment of the human/animal
   > body, the search has been carried out and based on the alleged
   > effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out. specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application. as follows:

> See additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant. this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying an additional fee. this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant. this International Search Report covers only those claims for which fees were paid. specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently. this International Search Report is restricted to the invention first mentioned in the claims: it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1 and 14 (both partially), 2-13 (all completely)

    An isolated nucleic acid encoding erythropoietin wherein the nucleic acid has one or more mutations in a non-coding region, and wherein the erythropoietin has altered biological activity.
    Erythropoietin encoded by these nucleic acids.
    Uses of these erythropoietin molecules, e.g., as pharmaceuticals or in therapy.

2. Claims: 1 and 14 (both partially), 15 (completely)

    An isolated nucleic acid encoding erythropoietin, comprising a mutation in the coding region encoding erythropoietin, the amino acid residue present in the corresponding position in wild-type erythropoietin being amino acid 101.
    A mutant erythropoietin protein, wherein the amino acid residue at the position 101 is alanine.
    Use of this erythropoietin mutant, e.g., as pharmaceutical or in therapy.

3. Claims: 1 and 14 (both partially), 16 (completely)

    An isolated nucleic acid encoding erythropoietin, comprising a mutation in the coding region encoding erythropoietin, the amino acid residue present in the corresponding position in wild-type erythropoietin being amino acid 103.
    A mutant erythropoietin protein, wherein the amino acid residue at the position 103 is selected from the group consisting of aspartate, alanine, glutamate, histidine and lysine.
    Use of these erythropoietin mutants, e.g., as pharmaceuticals or in therapy.

4. Claims: 1 and 14 (both partially), 17 (completely)

    As invention 2, but concerning amino acid 104.

5. Claims: 1 and 14 (both partially), 18 (completely)

    As invention 2, but concerning amino acid 105.

6. Claims: 1 and 14 (both partially), 19 (completely)

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

As invention 2, but concerning amino acid 108.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9402611	A	03-02-1994	EP	0667903 A	23-08-1995
			US	5614184 A	25-03-1997
WO 9533057	A	07-12-1995	IT	FI940106 A	27-11-1995
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